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EDITORIAL REVIEW

THE BIOASSAY OF VITAMIN K*

S. ANSBACHER

Division of Experimental Medicine, The Squibb Institute for Medical Research, New Brunswick, New Jersey

In vitamin research, rapid progress is made possible by accurate methods of detecting and measuring the active principle. In investigations on vitamin K, biological procedures have been relied upon almost exclusively and, as with other vitamins, they were of the preventive or curative categories.

PREVENTIVE BIOASSAYS

In the early studies from 1929 to 1935, the preventive type of bioassay was solely employed. Dam ('35) assayed food-stuffs by rearing chicks for 1 month on a vitamin K-deficient ration in which the test material had been incorporated. If the blood clotting times of the animals fed this diet were considerably shorter than those of the controls receiving the deficient ration, then the material was considered to contain vitamin K.

Almquist and Stokstad ('37 b) adopted Dam's method, shortened the test period to 2 weeks, and used clotting times of more than 30 minutes as criterion for a negative result. This technic was modified by Almquist et al. ('38) in that chicks were first reared on a deficient diet (Almquist and Stokstad, '37 a) for 1 week, then from 7 to 14 or 21 days on the supplemented ration. The assay was terminated after

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7 days of supplementary feeding, when a series of suitable groups was available receiving the "Almquist reference standard," a hexane extract of alfalfa to which no "unit" value was assigned.

After attempts to shorten the assay time had failed, Almquist and Klose ('39 a) described a third method, in which chicks were kept for 2 weeks on the supplemented diet following the initial week on the deficient ration, although spontaneous cures had been observed to occur frequently during the third week. Moreover, the simple clotting test was replaced by Quick's ('38) prothrombin determination showing less variability when carried out with whole blood than with plasma. However, according to Macfie et al. ('39), "determination of crude blood-clotting times appears to offer a better means of biological assay than determination of prothrombin times, contrary to the recent findings of Almquist and Klose."

The preventive method of Almquist and Stokstad ('37 b) was employed by Dann ('38) whose unit was described by Snell ('39): The Dann unit is the least amount per gram of a chick's body weight daily which is necessary to prevent prolongation of clotting time beyond normal limits during a 2-week assay period. Dann noted that less than 50% of her negative controls had markedly prolonged clotting times and that the incidence of hemorrhage in these groups was less than 20%. In order to minimize the influence of these adverse conditions, a clotting time of less than 1 minute was considered normal, although it may be even longer than 6 minutes (Schønheyder, '36 a; Cheney, '39). Later, Dann ('39) followed the example of Almquist and Klose ('39 d) by adopting an assay based upon a curative technic; thus, all investigators confirmed Schønheyder's ('36 b) result: "Vitamin K is best estimated by the curative method."

CURATIVE BIOASSAYS

The first curative bioassays were made by Schønheyder ('36 b) with a technic based upon the observation that "animals suffering from vitamin K deficiency became normal with

reference to clotting time in 3 days when given sufficient food containing vitamin K.'' The coagulation process was determined by a method similar to that of Quick ('38), but instead of measuring it as prothrombin time, the relation between the concentrations of the added clotting agent, which coagulate a plasma from a sick animal and normal plasma in 3 minutes at 40°C., was considered as index of the degree of deficiency. The Schønheyder unit of vitamin K is defined as the smallest daily dose of a test substance per gram of chicken for 3 days which is able to reduce the animal's S value, i.e., the degree of sickness, from over 1500 to 10. This unit is small, but when it is multiplied by 1000 it equals the amount of substance which is necessary to cure an animal with an S value of 1500 to 2000 and a weight of 333 gm. in the course of 3 days, the animal receiving one-third every day.

Schønheyder's method was revised by Dam and Glavind ('38) who determined the degree of sickness of the individual animals prior to the assay. The Dam unit of vitamin K is defined as that amount which is to be administered to the test animal per gram of body weight in order to reduce its value $R \geqq 200$ to 1, $R = \frac{K}{K_n}$; K equals concentration of the tissue extract necessary to cause clotting of the blood plasma in 3 minutes; K_n equals the corresponding concentration for normal plasma. Apparently Dam's ''R'' value (Dam and Glavind, '40) is the same as Schønheyder's ''S'' value divided by 10; it characterizes the degree of reduction of clotting power and is roughly 1 for normal chicks. Furthermore, a selected dried spinach powder, formed into tablets and stored under precautions securing stability, was chosen as a standard and found to contain 500 Dam units per gram.

Thayer et al. ('38) modified Schønheyder's assay by replacing the complicated coagulation technic with the simple clotting test. Arbitrarily, 10 minutes or less was considered normal, although earlier data (Thayer et al., '37) had indicated a range of 1 to 6 minutes. The Thayer unit is defined as that quantity of material required to reduce the clotting time of the blood of 50% of a group of ten or more chicks, which

had been reared for the 14 days immediately following receipt from the hatchery on a diet practically devoid of vitamin K, to 10 minutes within the 3 days of administration of the test material. In a subsequent paper, Thayer et al. ('39 a) introduced dosage-response curves found to be of value only when several groups of birds were used, as it was necessary to find the dosage, the administration of which produced a response that fell on the curve, preferably near its midpoint. These investigators (Richert et al., '40) emphasized also the necessity of ascertaining the response of each group of chicks to a standard, believing that the variability of the degree of deficiency of different lots was the reason for gross inaccuracies in the bioassay.

Ansbacher's ('38) report that chicks responded to vitamin K within a very short time, which according to Cheney ('40) may be less than 30 minutes, caused Thayer et al. ('39 b) to reduce their period of assay to 18 hours and to administer the test material in a single dose. The Thayer-Doisy unit is based upon the shorter test and its definition is the same as that of the Thayer unit except for the assay period.

Dann ('39) adopted the longer technic of Thayer et al. ('39 a), and Macfie et al. ('39) further prolonged the assay period by giving the test material divided into four equal portions on 4 successive days and killing the animals on the fifth day, i.e., after a no-dose period of 24 hours, for the determination of clotting and prothrombin times. This method was used also by Almquist and Klose ('39 d) after they had found irregularity in the results obtained with preventive methods.

A rapid curative assay was advocated by Ansbacher ('39) whose unit is defined as the minimum amount necessary to render the blood clotting time of the vitamin K-deficient chick, weighing 70 to 100 gm., normal within 6 hours after administration. He recommended that assays be made with a constant amount of menstruum, namely 0.10 ml., since the oil used as a medium for administering the test material appeared to interfere to a small but noticeable degree with the potency, an observation recently confirmed by Thayer et al. ('40).

Furthermore, he preferred to make use of chicks showing pronounced hemorrhages in addition to markedly prolonged clotting times, thus assuring a more uniform deficiency. Indeed, Tidrick et al. ('39) found that hemorrhages appeared to occur only when the prothrombin content had fallen below about 10% of the values seen in normal chicks of the same age.

The use of a heated diet in place of an extracted fish meal ration employed by most investigators was recently proposed by Ansbacher ('40 c). One of the major sources of K-vitamin synthesis (Almquist and Stokstad, '35), so markedly interfering with bioassays, is thus eliminated, and the test period may be safely prolonged from 6 to 18 or 24 hours in order to allow adequate time for the maximum action of the vitamin (Tidrick et al., '39).

The ingenious bioassay of Flynn and Warner ('40) employs rats, the common bile duct of which is ligated after 7 to 8 weeks' subsistence on a diet known to be low in vitamin K. The prothrombin content of the blood, measured by the two-stage method of Warner et al. ('36) and Smith et al. ('37), was found to fall to a level of 10 to 25% of normal within 72 hours following the operation, and the response to treatment, either by the intraperitoneal or intravenous route, was determined by observing the speed and completeness of the prothrombin restoration. The rats can be employed several times, since the normal prothrombin level, brought about by vitamin K therapy, is maintained at best for a few days, but they become unsuitable as soon as general debility and hepatic cirrhosis occur as a result of bile duct ligation.

VITAMIN K UNITS

The approximate relationships of various vitamin K units were estimated by Dam ('40), by Brinkhous ('40), by Riegel ('40), and Ansbacher ('40b) analyzed the bioassay methods for the purpose of evaluating 1 unit in terms of any other one (table 1). Dam's standard spinach preparation (Dam and Glavind, '38), containing 500 Dam units per gram, was found to have a potency of 1 Ansbacher unit in 40 mg., i.e., 25 units/gm.

Therefore, 1 Ansbacher unit equals 20 Dam or 20 Schønheyder units, the two latter ones being of the same order of magnitude.

A concentrate from alfalfa (Fernholz et al., '39), having a potency of 1 Ansbacher unit in 50 µg., was assayed by the 3-day curative test of Thayer et al. ('39 a) and found to contain 1 Thayer unit in 50 µg. Therefore, Ansbacher's unit equals Thayer's unit, an expected result, since Dam et al. ('38) had noted "that the clotting power may be rendered normal between 4 and 6 hours after one single intravenous injection. The amount of vitamin injected was not very far from that which would have resulted in normal clotting power if it had been given on 3 successive days with 1/3 on each day."

TABLE 1

Units of vitamin K

1 Ansbacher unit equals		
	0.16 ml. of the Almquist Reference Standard	
	20 (1938) Dam	
	12½ (1940) Dam-Glavind	
	½ (1938) Dann	
	1¼ (1939) Dann	units
	20 (1936) Schønheyder	
	1 (1938) Thayer	
	2 (1939) Thayer-Doisy	

Thayer et al. ('39 b) noticed that the Thayer unit equals 2 Thayer-Doisy units as shown by their statement: "Actually, with birds of the same degree of deficiency approximately twice as much vitamin is required for a 50% response by the 3-day procedure" than by the 18-hour assay. Therefore, Ansbacher's unit should be equal to 2 Thayer-Doisy units. Indeed, Ansbacher et al. ('39, '40) found that Thayer-Doisy's 18-hour test required only one-half of the vitamin material needed in Ansbacher's 6-hour assay, and Tidrick et al. ('39) confirmed this observation in a quantitative study on prothrombin restoration in chicks.

The Dann unit, called "Almquist unit" by Elliot et al. ('40), Greer ('40), and Fitzgerald and Webster ('40), was claimed to equal 37½ Dam units (Greer, '40; Fitzgerald and Webster, '40). Calculating with 20 Dam units per Ansbacher unit, the latter should be equal to about ½ (i.e., 0.533) Dann unit. In the definition of Dann's unit by Snell ('39) reference is made

to "gram of a chick's body weight." If "gram" were correct, 100 Dann, i.e., 200 Ansbacher units would be required by a 100-gm. chick; however, 1 Ansbacher unit is therapeutically effective in a 100-gm. chick and prophylactically only 0.2 Ansbacher unit is needed (Ansbacher, '40 a). Thus the Dann unit refers to "kilogram of a chick's body weight."

TABLE 2

Potency of methylnaphthoquinone and phylloquinone

AUTHORS AND THEIR CO-WORKERS	INSTITUTION	METHYLNAPHTHOQUINONE ANSBACHER UNITS PER MILLIGRAM			PHYLLOQUINONE STANDARD UNITS[1] PER MILLIGRAM
Almquist	U. of California	3	30	1700	262–290
Ansbacher	Squibb Institute	2000	2000	2000	250 [2]
Dam	U. of Copenhagen			2000	500
Dann	Abbott Labs.			2000	300–400
Doisy	U. of St. Louis	5	500	2000	333–500
Kamm	Parke, Davis & Co.			2000	420–480
Moll	Merck Labs. (Germany)			3000	
Sampson	Merck Institute	< 2	850	1700	333
Sjögren	Astra Labs. (Sweden)			1700	500
		July 1939	Sept. 1939	More recently	

[1] Methylnaphthoquinone = 1000 units/mg.   [2] Only 70 units/mg. in 6-hour test.

The Almquist et al. ('38) reference standard was found (Ansbacher, '40 a) to contain 1.56% of total solids, $2\frac{1}{2}$ mg. of which had a potency of an Ansbacher unit. Therefore, the activity of this standard preparation is 1 Ansbacher unit in 0.16 ml. (0.10 ml. according to Almquist and Klose, '40).

VITAMIN K STANDARD UNIT

Proposals for an international standard vitamin K preparation appear in the current literature and the use of 2-methyl-1,4-naphthoquinone for this purpose was first suggested by Thayer et al. ('39 d). The results obtained with this substance in the various laboratories are a particularly striking illustration of the erroneous results so frequently occurring in vitamin K assays. In order to facilitate an interpretation of the published data, expressed in different units and reference standard equivalents, they were converted into Ansbacher units (table 2).

The antihemorrhagic activity of this methylnaphthoquinone was first mentioned in the July, 1939 issue of the Journal of the American Chemical Society where the following potency data appear: 3000 units per gram according to Almquist and Klose ('39 b); 5000 units per gram reported by Thayer et al. ('39 c) who stated that the activity of this compound is relatively insignificant when compared with the natural vitamin K_1 or K_2; 2,000,000 units per gram found by Ansbacher and Fernholz ('39) who emphasized that methylnaphthoquinone is practically as active as vitamin K. Two months later, Fernholz and Ansbacher ('39) confirmed their discovery and Thayer et al. ('39 d) published revised data showing: "The potency of 2-methyl-1,4-naphthoquinone (Thayer-Doisy units) agrees with the value previously assigned to the natural vitamin K_1, namely 1000 units per milligram. These results also confirm the findings of Ansbacher and Fernholz." Simultaneously, Almquist and Klose ('39 c) disclosed that methylnaphthoquinone had been reassayed and found to have a potency of 30,000 units per gram, and that "it is by no means as active as vitamin K." Later, however, Almquist and Klose ('39 d) described the "amazingly high activity of the compound" and fully confirmed the data of Ansbacher and Fernholz. Tishler and Sampson ('39) indicated that a potency of less than 2000 units per gram had been found in their initial tests with this substance and stated: "Following the appearance of the extremely interesting report of Ansbacher and Fernholz, we reinvestigated the activity of 2-methyl-1,4-naphthoquinone, and we are now in complete agreement with them." Further confirmatory publications appeared more recently from Dann ('39), Hepding and Moll ('39), Sjögren ('39), Dam et al. ('40 a), Emmett et al. ('40), and Thayer et al. ('40) with the result that this compound is now recognized as the most potent substance with vitamin K-activity (Riegel, '40).

The testing of methylnaphthoquinone, the vitamin K standard, in the various laboratories has resulted in the publication of new data on the relationships of various units. Dam et al. ('40 a) claimed it to have a potency of 25,000,000 Dam units

per gram, and because Ansbacher found 2,000,000 units, Ansbacher's unit equals 12½ Dam units. Since previously a ratio of 1:20 had been found for these 2 units, Dam ('40) suggested that the difference may be explained by the fact that Ansbacher's method was a rapid one in which the rate of absorption may lower the results. However, an analysis of Dam's data permits the conclusion that the difference in the ratio of 1:20 to that of 1:12½ is due to a change in Dam's unit. The potency of phylloquinone (vitamin K_1) was 20 Dam units per microgram in the earlier reports of Dam et al. ('39) and of Karrer and Geiger ('39), but it is now only 12½ Dam-Glavind units per microgram (Dam et al., '40 b).

Apparently Dann's unit has also been modified. Dann ('39) found methylnaphthoquinone to have a potency of 2,500,000 Dann units per gram; calculating with 25,000,000 Dam units per gram, the Dann unit corresponds to 10 Dam units. Previously, Dann's unit was claimed to equal 37½ Dam units; indeed, Dann ('39) observed that 75 mg. of Dam's standard with 500 Dam units per gram gave a response of a Dann unit, i.e., 13.3 Dann units per gram and a ratio of 1:37½ for the two units. In the same publication appeared the methylnaphthoquinone data from which the ratio of 1:10 was calculated. Therefore, Dann dealt with two different Dann units; namely, ½ of the one and 1¼ of the other one corresponded to Ansbacher's unit.

Following the suggestion of Thayer et al. ('39 d) to assign a value of 1 standard unit to 1 μg. of 2-methyl-1,4-naphthoquinone, several authors expressed the antihemorrhagic potency of phylloquinone in terms of this unit (table 2). However, Ewing et al. ('39) proposed 2-methyl-1,4-naphthohydroquinone diacetate as a standard, because it is less sensitive to light than methylnaphthoquinone. In view of the fact that the latter compound was found to have clinically the same prophylactic and therapeutic action as concentrates of naturally occurring vitamin K, Macfie et al. ('39) advocated to dispense altogether with assays and a standard preparation, especially since "biological tests cannot be depended on always to give clear-cut results."

In conclusion, it seems desirable to emphasize that the chemistry of vitamin K has been rapidly elaborated in spite of the difficulties inherent in bioassays. The clinical data show conclusively that this vitamin is essential for the formation and activation of prothrombin. Synthetic prothrombinogenic substances, such as, e.g., the standard methylnaphthoquinone, have now become part of the armamentarium of the surgeon and pediatrician. Consequently, many a jaundiced patient may be safely subjected to operation, since with the advent of vitamin K therapy, he is no longer such a serious surgical risk, and many a baby may be saved from hemorrhagic disease of the newborn.

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THE EFFECT OF THE QUANTITY OF BASAL FOOD INTAKE UPON THE UTILIZATION OF VITAMIN A [1]

KATHLEEN DIETRICH MUELDER [2] AND EUNICE KELLY

*Department of Foods and Nutrition, Division of Home Economics,
Michigan State College, East Lansing*

In recent years there has developed a growing recognition of the importance of interrelations which undoubtedly exist among the various essential nutrients in a diet. The effect of individual constituents of a diet, for instance, on the utilization of vitamin A is a question which presented itself when the varying results obtained during the course of standardized biological assays for vitamin A were scrutinized. Numerous explanations of the causes of these variations in the response of rats under controlled conditions have been offered. Homogeneity of the colony from which the test animals have been selected, percentages of fat in the basal diet, and the quantity of basal ration consumed during the depletion period are factors named by Nelson and Swanson ('31) as affecting vitamin A determinations. Honeywell et al. ('31) reported that variations in yeast as a source of the B complex and the possibility of traces of vitamin A from yeast caused differences in the response of animals during a vitamin A assay. It has been suggested further by Javillier and Emerique ('31) that an adequate supply of vitamin D and the criteria for judging the state of depletion are important factors in the biological estimation of vitamin A.

[2] Formerly Assistant Professor of Foods and Nutrition and Research Assistant in Foods and Nutrition at Michigan State College, East Lansing, Michigan.

Such observations by investigators carrying out the accepted technique for vitamin A determinations, as well as a general interest in the possible physiological interrelationships of dietary factors, suggested this study of the influence of different constituents in the basal ration upon the utilization of vitamin A. The problem was undertaken not from the point of view of its relation to the technique for assays but rather from the standpoint of its relation to vitamin A metabolism. The first phase of this experiment was planned to test the influence of the caloric intake from basal ration consumed during the test period upon the utilization of vitamin A, using rats with the same nutritional background and with similar inheritance. Rate of growth and incidence of ''abscessed'' areas were the criteria used to measure the response, to graduated levels of vitamin A, of animals consuming restricted, as compared with unrestricted, quantities of the basal ration. The data include the responses of 237 animals and were collected over a period of 2 years, 1935–1937.

<div align="center">PROCEDURE</div>

The basal vitamin-A-deficient ration employed was essentially that recommended for assay according to the U. S. Pharmacopeia, XI Revision ('35). In order, however, to insure a sufficiency of the B vitamins at the restricted levels of food intake, the dried bakers' yeast was not incorporated into the ration, but a 0.5 gm. portion was fed daily as a separate supplement. Triads of animals (Haag, '34; Smith, '34) selected on a basis of age, weight, sex, and litter, were depleted on this ration.[3] A uniformity in weight range in a triad during the depletion period was maintained by controlling the food intake within the group. At the end of the depletion period one animal from each triad was selected as a negative control, one as a calorie control, and one as a positive control.

Since the food consumption of the calorie control rats was determined by that of the negative control animals, a possible

[3] Composition of basal ration: 18% vitamin-A-extracted casein, 4% Osborne and Mendel salt mixture, 73% irradiated corn starch, and 5% fat.

means of prolonging the survival time of the latter was sought in order to insure greater accuracy in the paired-feeding. Sherman and Storms ('25) demonstrated that an increase in age and in weight of the animals at the time of starting on a vitamin A deficient ration will cause a relatively small increase in depletion time but also a longer time of survival after depletion. These findings were used as a guide in determining the age and weight for starting the animals on experiment. The rats, weaned at an age of 25 to 28 days and having a weight of 50 to 55 gm., were continued on the stock ration [4] for approximately 2 weeks. In actual practice, the animals were started on the vitamin-A-deficient basal ration at an age of 39 to 43 days. The range in weight was 105 to 115 gm. for the males and 90 to 100 gm. for the females.

Body weight and food consumption were recorded weekly for the first 3 weeks, and twice a week for the remainder of the depletion period. Examinations for signs of ophthalmia were made at the same times. The criteria for deciding when a triad reached depletion were failure to gain in weight for 1 week and evidence of ophthalmia.

Oil dilutions [5] of reference cod liver oil (U. S. Pharmacopeia, XI) were prepared each week quantitatively, so that when fed from calibrated droppers, 2 drops of solution carried 1, 3 or 6 U. S. P. XI Units of vitamin A. This supplement was fed daily, the negative control animals being fed 2 drops of refined cottonseed oil. The positive and negative control animals were allowed the basal ration ad libitum during the experimental period. The calorie control rats were restricted to an allowance of basal ration equal to that consumed by the negative controls. The calorie control rat, however, in any one triad, was fed the same amount of the cod liver oil supplement (1, 3 or 6 International Units of vitamin A) as

[4] Composition of stock ration in grams: whole milk powder 300, whole wheat 500, oats 500, whole yellow corn 480, flaxseed oil meal 200, calcium carbonate 20, Kelco meal (containing seaweed) 80. On alternate days each animal receives 2 to 3 gm. raw lean beef and 2 to 3 gm. raw leaf lettuce.

[5] Refined cottonseed oil was used for this purpose as well as to furnish the 5% fat in the basal ration.

was given the positive control rat of the same triad. Because of the number of animals used in this experiment and the fact that the tests were run over a 2-year period, the supply of reference cod liver oil had to be replenished several times. Variations in vitamin A content of the different reference samples should not, in this study, modify the interpretation of results, because comparisons are made between groups of animals in which the calorie control and positive control from the same triad were always fed the cod liver oil supplement from the same dilution.

During the 4-week experimental period the animals were weighed weekly. The food consumption records for the positive controls were also made weekly; but daily weighings of the food consumed by the negative controls were recorded because these quantities were used as the basis for feeding the calorie controls. The average survival time of all negative controls of the triads was 21.2 days, 21.0 days and 22.6 days respectively for the 1-, 3- and 6-unit triads with a range in survival time of 13 to 28 days. In those triads in which the negative control animal died before the twenty-eighth day, food allowance for the calorie control was determined by the average food consumption of negative animals of other triads in corresponding periods of the experiment.

Complete gross autopsy findings were recorded, although it was found that, for the purposes of comparison desired in this study, an enumeration of the number and location of proliferated areas of keratinized epithelial tissues furnished a useful, objective indication of the extent of vitamin A deficiency. Vaginal smears were made thrice weekly during the entire experiment. These data it was hoped would give an additional basis for interpretation of the findings, since the vaginal epithelium has been found to be sensitive to a vitamin A deficiency (Aberle, '33; Mason and Ellison, '35). It was found, however, that this test was too sensitive for the purpose of this work. Recovery from persistent keratinization, for instance, was too rapid to serve as a basis for differentiating the response to the same level of vitamin A supplement of

rats on restricted versus unrestricted intake of the basal ration.

DISCUSSION OF RESULTS

From table 1, in which are given summarized data (arithmetic means) for the depletion period, it may be seen that the manner of selecting triads of animals and the method of maintaining weight uniformity within triads during the depletion period resulted in uniform groups of animals at the beginning of the test period.

TABLE 1

Average ages, weights, and food consumptions of animals from weaning to end of depletion period on the vitamin A deficient diet

GROUP [1]	YEAR	TRIADS		WEIGHT AT WEANING [2]	DEPLETION PERIOD				
		Male	Female		Time	Age at begin-ning	Weight at		Weekly food con-sumption
							Begin-ning	Ending	
		No.	*No.*	*gm.*	*weeks*	*days*	*gm.*	*gm.*	*gm.*
1	1935–36	6	7	54.5	7.6	41.8	103.9	174.8	67.8
2	1936–37	7	7	58.4	8.3	39.9	103.2	180.7	76.4
3	1935–36	6	7	55.7	8.2	41.5	102.3	177.3	67.7
4	1936–37	6	4	60.4	8.2	40.2	107.1	189.0	78.5
5	1935–36	6	6	54.1	8.0	41.6	102.1	177.6	67.7
6	1936–37	6	5	57.9	7.8	40.5	102.9	176.8	75.6

[1] Groups 1 and 2 were put on supplements of 1 I.U. of vitamin A; groups 3 and 4 on 3 I.U., and groups 5 and 6 on 6 I.U.

[2] Average age at weaning was 27.9 days.

Since negative and calorie control rats of a given triad consumed the same quantity of basal diet but the negative control rat was denied vitamin A supplement, the differences in response of these animals was a direct measure of the effect of the vitamin A fed. Also, since calorie and positive control rats in a triad received the same level of vitamin A supplement but consumed different quantities of basal diet, differences in response of these animals measured the response due to variations in food intake.

Effect upon growth. In table 2 are given the summarized data for the 4-week experimental period for both sexes combined for the 2 years. A statistical analysis of weekly food

consumptions, weight changes, and number of "abscesses" evident at autopsy was calculated for each year and each sex, separately as well as combined, in order to determine if there was consistency in response during the 2 years. Since the

TABLE 2

Average weekly food intakes and weight changes during 4-week experimental period and "abscesses" evident at autopsy

YEAR AND DESCRIPTION OF ANIMALS	ANIMALS	LEVEL OF VITAMIN A	AVERAGE WEEKLY FOOD INTAKE [1]	AVERAGE WEEKLY WEIGHT CHANGE [1]	"ABSCESSES" EVIDENT AT AUTOPSY [2]
	No.	*Units* [3]	*gm.*	*gm.*	*no.*
1935–37 males and females					
Negative	27	0	41.0 ± 2.01	—12.2 ± 1.49	3.2 ± 0.25
Calorie	27	1	42.4 ± 1.44	— 8.3 ± 1.02	1.3 ± 0.26
Positive	25	1	53.6 ± 1.37	— 2.8 ± 1.11	1.7 ± 0.27
1935–37 males and females					
Negative	23	0	41.9 ± 2.38	—14.3 ± 1.67	3.5 ± 0.30
Calorie	23	3	43.9 ± 1.52	— 7.3 ± 1.09	1.3 ± 0.19
Positive	22	3	65.6 ± 1.33	+ 5.6 ± 0.63	1.0 ± 0.22
1935–37 males and females					
Negative	23	0	36.9 ± 2.43	—16.3 ± 1.61	3.7 ± 0.25
Calorie	23	6	40.8 ± 1.52	— 8.1 ± 0.98	1.4 ± 0.21
Positive	23	6	70.8 ± 1.67	+ 8.0 ± 0.68	0.9 ± 0.19

[1] Arithmetic mean ± standard error.
[2] Areas that most frequently showed "abscesses" were middle ear, base of tongue, lymph glands in neck, salivary glands, and genito-urinary tract.
[3] International Units.

2 years' response was similar and, for the purpose of discussion, the mean response of the combined groups will be considered, only the summarized data for the combined groups are presented.

There was increasing difficulty at each higher level of vitamin A intake in keeping the calorie control group adjusted to the low level of food consumed by the negative control group. A slight increase in caloric intake at each increase in level of vitamin A might be expected because of increased appetite

and improved general well-being in the calorie control rats receiving some vitamin A. Furthermore, failure of the negative control rats to consume the basal diet during the last days of survival caused some of the differences observed.

In table 3 are given the average weekly differences in response of all seventy-three calorie control animals as compared with all negative control animals. During the 4-week experimental period, the twenty-seven calorie control rats given 1 unit of vitamin A lost 3.9 gm. less per week than did the corresponding twenty-seven negative control rats. This decreased loss, which, therefore, might be considered a "gain" of 3.9 gm. over that of the negative control animals accompanied an increase in caloric intake of but 5.7 calories from

TABLE 3

Comparison of weight change and food intake in negative and calorie control animals

VITAMIN A LEVEL	NUMBER OF TRIADS	DIFFERENCE IN			EXPECTED GAIN IN WEIGT DUE TO DIFFERENCE IN CALORIE INTAKE
		Weight change per week	Food intake per week	Calorie intake per week	
I.U.[1]		gm.	gm.	Cal.	gm.
1	27	3.9	1.4	5.7	+0.14
3	23	7.0	2.0	8.18	+0.21
6	23	8.2	3.9	15.95	+0.40

[1] International Unit.

1.4 gm. of food. This increase of food would not have been expected to give more than 0.14 gm. gain in weight per week, if calculated on the basis of grams gained per total calories ingested. This difference in weight of 3.9 gm. per week for the 4-week period, then, may be considered a measure of the response to the 1 unit of vitamin A administered. There was an increase of 3.1 gm. gain at the 3-unit level over that of the 1-unit level, and 1.2 gm. gain at the 6-unit level over that of the 3-unit level which was greater than can be accounted for by the slight increase in food intake. This observed "leveling off" of weight increases would indicate that the 3 and 6 unit levels of vitamin A were beyond the most sensitive areas of a curve of response of the animals used in these experiments.

In table 4 are given the differences in average weekly gain in weight of all positive control rats over that of the calorie control rats of corresponding triads. Since the calorie control rats and positive control rats were receiving identical doses of vitamin A supplement (1, 3 or 6 units), in any one group, but consuming different quantities of food, differences in average gain in weight between the two groups of animals were to be explained on the basis of differences in food intakes. There was an increased gain in weight which was directly related to the increased caloric intake at each succeedingly

TABLE 4

Comparison of weight change and food intake in calorie and positive control animals

VITAMIN A LEVEL	NUMBER OF TRIADS	DIFFERENCE IN			RATIO OF DIFFERENCE IN WEIGHT CHANGE TO CALORIE INTAKE	GAIN IN WEIGHT PER CALORIE [2]
		Weight change per week	Food intake per week	Calorie intake per week		
I.U.[1]		gm.	gm.	cal.		gm.
1	27	5.5	11.2	45.8	0.120	N—0.073 C—0.048 P—0.013
3	23	12.9	21.7	88.75	0.145	N—0.083 C—0.036 P+0.021
6	23	16.1	30.0	122.7	0.131	N—0.108 C—0.048 P+0.028

[1] International Unit.
[2] N = negative control animal; C = calorie control animal; P = positive control animal.

higher level of vitamin A. The ratio of the weight increase to the calorie increase (column 6, table 4) however, was strikingly similar for each level of vitamin intake. It would seem that there was improvement in general well-being and perhaps even in appetite (Sampson, Dennison and Korenchevsky, '32) which induced increased caloric intakes and gains in weight with increased levels of vitamin A.

Nelson and Swanson ('31) have reported that the quantity of basal ration consumed during the depletion period is the measured variable having the greatest percentage effect on

weight gains in a vitamin A assay. In this experiment the food intake of each triad was adjusted during the depletion period so as to keep each member of the triad gaining at a uniform rate and thus have an even group at the end of the depletion period. It will be seen (table 4) that during the experimental period, also, the caloric intake was an important factor in producing weight changes, the relative proportion of weight increase differing at different levels of vitamin intake. Expressed as percentage, 59% of the gain in weight of the positive control group over that of the negative control group was due to increased calories at the 1-unit level, 64% at the 3-unit level, and 66% at the 6-unit level of vitamin intake. A part of the discrepancies in the literature in respect to weight responses with vitamin A assays may be due to differences in food intake. It has been calculated from these data that a gain of 0.025 gm. per week for each calorie ingested might be expected in the response of animals evenly balanced as to sex on this experimental diet.

Although not indicated in the tables, males demonstrated a slightly higher gain in body weight per calorie of food ingested than did females. This is in agreement with the findings of Palmer and Kennedy ('31) on sex differences in gain in weight and illustrates the necessity of having the groups evenly matched in respect to sex when using weight as a criterion of differences in response.

All of these data have been tested by the "t" tests for significance of differences in mean response according to the formula $t = \frac{M - M_1}{\sqrt{\frac{(\sigma)^2}{N} + \frac{(\sigma_1)^2}{N_1}}}$, and were found to be highly significant. That is, the "t" values for the mean differences in response between the negative and calorie and between the calorie and positive groups were high.

Effect on incidence of pus-like accumulations in organs and tissues (abscesses). Another criterion for judging vitamin A utilization was the presence or absence of areas of pus-like material in organs, glands, and tissues as examined grossly at autopsy. It is recognized that, if these areas were typical

of vitamin A deficiency, they probably were in most instances accumulations of keratinized epithelial cells (Wolbach, '37). For convenience, these pus-like accumulations have been tabulated as "abscesses" evident at autopsy. It is seen that the negative control animals at each level of vitamin administration presented essentially the same picture in respect to number of "abscesses," namely, 3.2 for the 1-unit group, 3.5 for the 3-unit group, and 3.7 for the 6-unit group, showing the uniformity of negative groups in developing "abscesses" when deprived of vitamin A on this experimental diet.

Administration of even 1 International Unit of A reduced the number of areas of epithelial proliferation at least 50%.

The use of 6 units of vitamin A did not result in any significant improvement over 1 unit of vitamin A in preventing occurrence of "abscesses" seen at autopsy. However, the general appearance of the 6-unit group was better than that of the 1-unit group. At the end of the 4-week experimental period, there was no external evidence of ophthalmia among the group receiving 6 units of vitamin A. It is believed by Coward ('38) that four or five times as high levels of vitamin A intake are necessary to obtain a removal of all evidence of ophthalmia as are required to stimulate gain in weight. It is possible, in the light of the results of this study, that the administration of even more than five times the quantity of vitamin A required to produce weight gains is necessary to prevent all evidence of ophthalmia. While results from these experiments indicate a definite reduction of "abscesses" by the administration of vitamin A, analyses of separate groupings of animals indicate considerable variation in response at different levels of supplement, between sexes, and between the 2 years of the study. This conclusion concerning the effect of vitamin A intake upon incidence of "abscesses" has been substantiated by the results of the "t" tests for significance of difference in mean response. In all cases the "t" values for the mean difference in response between the negative and calorie control animals or between the negative and positive control animals were high, indicating

a highly significant reduction in "abscesses" by administration of the vitamin.

The "t" values for the mean difference in response due to calories ingested between the calorie and positive control animals were small, showing that the caloric intake had no statistically significant effect upon the incidence of "abscesses." However, it will be noted that with the calorie control groups there was no reduction in incidence of "abscesses" by the administration of 6 units of vitamin A as compared with 1 unit of vitamin A (1.4 and 1.3 "abscesses" respectively), while with the positive control groups and similar increases in vitamin A there was a reduction in number of such areas from 1.7 to 0.9. Although this difference in incidence of "abscesses" reached only the borderline of significance (there was a "t" value of 2.29 whereas a "t" value of 2.67 would have been required for statistical significance) there was some indication of improved metabolism with an unrestricted when contrasted with a restricted food intake when the higher levels of vitamin A were administered.

SUMMARY

The influence of the quantity of basal food intake on vitamin A utilization has been studied by means of carefully controlled triads of rats as measured by rate of growth and incidence of "abscessed" areas. For the levels of vitamin A used in this experiment, the caloric intake was responsible for a greater proportion of the gain in weight during a 4-week period than was the unitage of vitamin A administered, the percentage of the gain in weight from caloric intake increasing at each elevation of vitamin intake. When the intake of vitamin A was identical in different groups of animals, the gain in weight was directly related to the quantity of basal diet consumed during the period of observation.

There was a highly significant reduction in number of "abscessed" areas by the administration of either 1, 3 or 6 International Units of vitamin A below the number of such areas exhibited by the negative groups, but no statistically

significant difference between the levels of vitamin used in this experiment. The quantity of basal food intake showed no statistically significant influence upon incidence of "ab scessed" areas. However, there was some indication of an advantage of unrestricted as compared with restricted food intake at the highest level (6 units) of vitamin A intake.

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THE HEAT PRODUCTION AND BLOOD AND URINE CONSTITUENTS AFTER ADMINISTRATION OF 1(—) HISTIDINE TO THE DOG [1]

A. G. EATON AND J. R. DOTY

Department of Physiology, Louisiana State University,
School of Medicine, New Orleans

Standard textbooks and reviews generally credit histidine with no specific dynamic action. The basis for these statements appears to be a single experiment performed by Rapport and Beard ('28) in which they administered 5 gm. of histidine dihydrochloride by stomach tube to a dog and observed no increase in heat production during the next 3 hours. If one considers that approximately one-third of the 5 gm. administered was hydrochloric acid, it is easy to see that a small rise in heat production, following the feeding of so small an amount of histidine, might not be apparent. Furthermore, the dihydrochloride of histidine is a very acid substance and might easily produce conditions within the gastro-intestinal tract which might delay absorption. Nor has the effect of the administration of this quantity of hydrochloric acid upon the heat production been adequately evaluated. Thus, the effect of a small amount of histidine, absorbed over a prolonged period, might not be observable.

Reinwein ('28) was unable to obtain increases in the oxygen consumption of rats, guinea pigs and liver slices following histidine administration but upon the administration of a single dose of 25 gm. of histidine to a 13.5 kg. dog did observe

an average increase of 19.9% in the heat production. He calculated the increase to be 7.2 Cal. per gram of administered nitrogen. However, since he gave no data regarding either the excretion of the compound or the quantity of urea formed from it, we are unable to make a direct comparison of his results with ours.

Not only were we interested in arriving at an accurate measure of the specific dynamic action of histidine but we wished to determine the rate of excretion, deamination and urea formation following its intravenous injection. It was hoped that these data together with previously published figures for glycine, lysine and arginine (Doty and Eaton, '39; Eaton and Doty, '39) might throw some light on the theory of urea formation of Krebs and Henseleit ('32) as applied to the intact normal dog.

METHODS

With but few exceptions the plan of experiments and the methods of analysis have already been described (Doty and Eaton, '39). Urinary amino nitrogen was determined by the manometric method of Van Slyke ('29) upon the silver histidine precipitate prepared according to Block ('34).

It was planned to give histidine monohydrochloride in amounts practically equal to the quantities of arginine monohydrochloride and lysine monohydrochloride administered in former experiments so that a fair basis of comparison might be obtained. However, a preliminary trial showed the monohydrochloride of histidine to be unsuitable for satisfactory intravenous injection. In one experiment reported in this paper, the monohydrochloride (9.6 gm.) was carefully neutralized to pH 7.0 (glass electrode) by means of sodium hydroxide and the resulting solution injected intravenously. Since we wished, however, to avoid possible complications resulting from the injection of a comparatively large quantity of sodium chloride we resorted to the use of the l(—)histidine (free base). The four remaining experiments included in the tables were performed by dissolving the histidine (free base) in

approximately 110 cc. of physiological saline, sterilizing by heat, and injecting the resulting solution into a saphenous vein at a constant rate during a period of 10 minutes. Because restlessness during several attempted experiments had prevented us from following the heat production accurately, we performed the two experiments on dog 6 H and the one on dog 7 H under deep and constant sodium amytal anesthesia in a room with the temperature sufficiently high to maintain the dog's temperature at the normal level.

Preparation of free histidine base. This compound was readily prepared from a saturated aqueous solution of the monohydrochloride by slowly adding an amount of a concentrated aqueous solution of lithium hydroxide exactly equivalent to the hydrogen chloride. Precipitation of the histidine began almost immediately and was rendered more complete by adding an equal volume of 95% ethyl alcohol and by cooling the mixture overnight in a refrigerator at 0°C. The crystals were collected on a glass filter, freed of the mother liquor by suction and thoroughly washed with 95% alcohol. Nearly theoretical yields of histidine were obtained. When necessary, the last traces of lithium chloride can be removed by solution of the histidine in the minimum amount of hot water and by reprecipitation as above with 95% alcohol.

RESULTS

The results of blood analyses, as well as average comparative values for intravenous experiments with glycine, lysine, and arginine, compiled from previously published papers (Doty and Eaton, '39; Eaton and Doty, '39), are shown, for rapid comparison, in table 1. Figures for heat production and urinary nitrogen fractions are included in table 2.

The blood amino nitrogen changes after histidine administration tend to parallel those obtained when arginine was injected, except that the initial decrease is somewhat faster for arginine. At least a partial explanation for this difference is obvious when one examines table 2. The average excretion of histidine is only 1.44 gm. in contrast to 2.92 gm. for arginine.

Since the fastest rate of excretion occurs when the concentration in the blood is at a high level, it is very probable that the initial rate of excretion of arginine is about twice that of histidine. The fall in blood amino N after lysine is due almost entirely to excretion of that compound into the urine, while

TABLE 1

Blood amino nitrogen and blood urea nitrogen values after intravenous administration of histidine

DOG NO.	ADMINISTERED GRAMS	MILLIGRAMS PER 100 CC. OF BLOOD						
		Control period (hours before administration)		Experimental period (hours after administration)				
		1	0.5	1	2	3	4	5
Amino nitrogen								
5	9.6 gm. as monohydrochloride (neutralized)	8.1	8.3	19.1	15.4	14.0	11.6	11.2
5	8.1 gm. as free base in 0.85% NaCl	11.3	11.3	17.4	16.0	14.8	13.6	12.9
5	7.9 gm. as free base in 0.85% NaCl	7.4	7.5	15.4	14.3	11.5	10.3	9.3
6 H	8.1 gm. as free base in 0.85% NaCl	7.3	7.3	17.8	13.7	11.9	10.4	10.3
6 H	8.1 gm. as free base in 0.85% NaCl	7.9	7.4	19.8	15.4	12.8	10.7	10.0
Average for histidine		8.4	8.4	17.9	15.0	13.0	11.3	10.7
Average for glycine (Doty and Eaton, '39)		9.8	9.5	29.4	13.9	11.2	11.6	10.8
Average for lysine (Doty and Eaton, '39)		9.7	9.5	19.7	13.5	12.0	12.0	12.5
Average for arginine (Eaton and Doty, '39)		10.2	10.2	17.0	12.6	10.4	9.9	10.0
Urea nitrogen								
5	9.6 gm. as monohydrochloride (neutralized)	8.9	8.1	10.5	13.2	15.0	15.5	15.3
5	8.1 gm. as free base in 0.85% NaCl	9.0	9.0	10.2	12.6	15.3	16.7	15.2
5	7.9 gm. as free base in 0.85% NaCl	9.5	9.9	11.0	13.8	15.9	15.4	14.8
6 H	8.1 gm. as free base in 0.85% NaCl	7.2	7.7	9.3	10.1	11.6	10.2	8.9
6 H	8.1 gm. as free base in 0.85% NaCl	10.5	10.6	12.8	14.7	14.6	13.8	12.3
Average for histidine		9.0	9.1	10.8	12.9	14.5	14.3	13.3
Average for glycine (Doty and Eaton, '39)		11.6	12.0	15.7	19.3	19.9	17.6	15.9
Average for lysine (Doty and Eaton, '39)		12.9	13.6	13.1	13.8	13.1	12.2	11.5
Average for arginine (Eaton and Doty, '39)		11.0	11.1	15.4	18.5	20.9	20.1	18.5

the rapidity of removal of glycine from the blood stream represents not only an excretion comparable to that found with arginine, but also an extremely rapid rate of deamination.

The explanations given above are further confirmed by the values for blood urea nitrogen also shown in table 1. The

average changes in the blood urea nitrogen levels due to the administration of histidine roughly parallel those due to arginine. The value with arginine, however, does rise a little more rapidly than with histidine and remains somewhat higher at the end of the period. It should be stated that dog 5 was used for both histidine and arginine experiments, which would seem to make comparisons more nearly valid. The blood urea nitrogen values for glycine almost exactly parallel those for arginine for the first 3 hours but fall off more rapidly during the final 2 hours of the experiment. The figures obtained after lysine administration show no rise outside the limits of accuracy of the method and show a considerable decrease below control levels during the last 3 hours.

In only one (dog 7 H) of the six experiments shown in table 2 did the excretion of histidine equal that found with the other amino acids. Since, also, in this experiment alone, the extra urinary urea nitrogen appeared to exceed the extra total non-histidine urinary nitrogen, one must not take this amino acid figure too seriously. It is entirely possible that some forms of nitrogen, other than histidine nitrogen, might have been carried down along with the silver precipitate. Apparently histidine is more readily reabsorbed by the kidney tubules than is lysine or arginine. This conclusion is in accord with the finding that when these three compounds are fed under comparable conditions, histidine is the most rapidly absorbed from the gastro-intestinal tract (Doty and Eaton, '37).

The administration of histidine (with the one exception noted above) resulted in the excretion of a considerable amount of nitrogen in the urine which is neither urea nitrogen nor histidine nitrogen. A few determinations showed that no appreciable quantity of imidazole compounds other than histidine was excreted. The ammonia excretion was small and could account at most for only a small fraction of this undetermined nitrogen. In marked contrast to histidine, arginine gave very little undetermined nitrogen.

Because the blood urea nitrogen values had not returned to control level at the end of the experimental period, it is very

TABLE 2

Heat production and urinary nitrogen after intravenous administration of histidine

DOG NO.	WEIGHT	CONTROL PERIOD		EXPERIMENTAL PERIOD		URINE UREA N	EXCESS URINE UREA N	EXCESS TOTAL N (LESS N OF AMINO ACID EXCRETED)	AMINO ACID EXCRETED	AMINO ACID METABOLIZED	CAL./GM. EXTRA N	SPECIFIC DYNAMIC ACTION
		Average B.M.R.	Basal urine urea N	Amino acid used	Average metabolic rate							
	kg.	Cal./hr.	mg./hr.	gm.	Cal./hr.	mg./hr.	mg.	mg.	gm.	m/Mol.		Cal./m Mol. amino acid metabolized
5	10.5	16.55	55.8	9.6 gm. as histidine monohydrochloride neutralized with NaOH	17.79	132.1	406	665	0.78 as free base	9.66	9.34	0.642
5	10.7	—	66.4	8.1 gm. free base in 0.85% NaCl	—	140.4	411	726	0.66 as free base	9.79	—	—
5	11.1	—	70.5	7.9 gm. free base in 0.85% NaCl	—	168.4	612	967	0.78 as free base	14.57	—	—
6 H	7.3	16.77	57.4	8.1 gm. free base in 0.85% NaCl	17.76	169.2	604	1067	1.45 as free base	14.38	4.67	0.347
7 H	7.0	13.71	107.3	8.1 gm. free base in 0.85% NaCl	15.17	219.9	605	524	3.25 as free base	14.39	13.97	0.508
6 H	6.4	13.91	55.5	8.1 gm. free base in 0.85% NaCl	15.10	152.5	542	844	1.75 as free base	12.90	7.04	0.460
Average							530	799	1.44		8.75	0.489
Average for glycine (Doty and Eaton, '39)							714	961	3.02	51.0	12.91	0.244
Average for lysine (Doty and Eaton, '39)							458	—	6.65	16.4	—	—
Average for arginine (Eaton and Doty, '39)							182	185	2.92	3.25	22.35	0.399

difficult to estimate the amount of urea actually formed from the histidine administered. A rough approximation of the urea formed from the administered histidine but remaining in the tissues at the end of the experimental period may be made by applying the calculations used by Eaton, Cordill and Gouaux ('37). They established the basal rate of urea excretion previous to total nephrectomy and followed the increase of urea in the blood during a 5-hour experimental period. Calculations, making use of the weight of the dog, the rate of accumulation of urea nitrogen in the blood and the basal urea nitrogen production, indicated that the blood urea nitrogen was in equilibrium with approximately 70% of the body tissues of the dog. If one applies the same calculations to the histidine experiments, here reported, one finds the average urea nitrogen retention to be 240 mg. per dog experiment. This added to the average of 530 mg. per dog experiment excreted in the urine would give a total of 770 mg. of urea nitrogen formed from histidine. The amount of alpha amino nitrogen in the average quantity of histidine administered and retained was 593 mg. per dog. From this calculation it would appear that approximately 80% of the urea nitrogen formed could have come from simple deamination of the histidine molecule. Since the amount of undetermined (non-imidazole) nitrogen is large, it is further apparent that the imidazole ring was broken to some extent during the 5-hour period. This is more evident when one adds the calculated retained urea nitrogen in each experiment to the total non-histidine nitrogen excretion. The average per dog experiment of 1038 mg. (maximum 1278 mg.) nitrogen which could be accounted for in this fashion is well in excess of the average 593 mg. of alpha amino nitrogen administered but not excreted as such in the urine but comes far from accounting for the average of 1779 mg. of total histidine nitrogen administered and retained for metabolism. Evidently only a little more than half of the retained histidine nitrogen was transformed into urea or excreted as undetermined N within the 5-hour experimental period.

It is of interest to apply the same calculation to our results on arginine already published ('39) in order to have a more extensive basis of comparison of the metabolism of these two basic amino acids. The average total amount of arginine nitrogen administered, after deduction of that excreted in the urine, was 1852 mg. Half of this or 926 mg. should be transformed very rapidly into urea nitrogen by the action of arginase. The average excretion of urea for the 5-hour period amounted to only 182 mg. and the average amount calculated as formed but retained in the tissues would be 482 mg. per dog experiment. The total of 664 mg. as a rough approximation would thus fall far short of the quantity of urea to be expected by the primary action of arginase. These results would seem to have a significant bearing upon a consideration of the Krebs and Henseleit theory as regards the participation of the arginine-ornithine-citrulline cycle in normal urea formation. Although a final interpretation of the results with arginine must await a similar study with ornithine, we are probably justified in assuming that there is no significant accumulation of ornithine in the blood. Each molecule of ornithine contains two amino groups whereas each molecule of arginine contains only one such group available for the formation of nitrogen gas in the Van Slyke procedure. Similarly, the transformation of arginine to citrulline would involve no reduction in the amino nitrogen content of the system. It is conceivable, then, that the participation in the Kreb's cycle of a substantial fraction of the administered arginine would result in a slower rate of fall of the blood amino nitrogen as compared with the fall observed after administration of other amino acids. Our experiments furnish no evidence of such an effect. It is obvious, of course, that our results with arginine may require reinterpretation in the light of the suggestions that arginine may be metabolized after becoming an integral part of a protein molecule (Dirr, '39; Schmidt, Allen and Tarver, '40).

The over all change in the production of urea from the amino nitrogen of glycine or from histidine amino and non-amino nitrogen appears to take place more rapidly than does the

hydrolytic transformation of the guanido group of arginine into urea. It would seem improbable, therefore, that under the conditions of our experiment the latter mechanism is quantitatively important in the production of urea from glycine or histidine.

We must most emphatically state that our experiments do not disprove the theory of Krebs and Henseleit. They do, however, indicate that the urea formed from arginine, under the conditions used by us, could be formed entirely by mechanisms which do not involve either citrulline formation or a new synthesis of arginine. Regardless of any theoretical considerations, we have shown that more histidine is metabolized than arginine during a 5-hour experimental period when the two amino acids are administered in nearly equimolecular quantities.

Histidine exhibits a specific dynamic action about 50% greater than arginine but only about half that of an equal weight of glycine. On the basis of millimols deaminized the specific dynamic action of histidine is about twice that of glycine.

SUMMARY

Upon intravenous injection of 1(—)histidine into the dog there is a prompt and long sustained rise in the urea nitrogen of the blood. In contrast to the results with glycine, lysine and arginine, there is but little excretion of unchanged histidine into the urine. Histidine is metabolized slightly faster than arginine as shown by the greater excretion of urea during the 5-hour experimental period.

Histidine exhibits a specific dynamic action which is about 50% higher than that shown by arginine, both on the basis of amount administered and amount metabolized. The total increase in heat production is about half as great as that produced by an equal weight of glycine.

No toxic effects of histidine were noted.

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ON THE MANNER OF ACQUISITION OF FLUORINE BY MATURE TEETH [1]

MABEL W. PERRY AND W. D. ARMSTRONG

The Laboratories of Dental Research and Physiological Chemistry,
The University of Minnesota, Minneapolis

The demonstration (Armstrong, '37; Armstrong and Brek-hus, '38 b) of significantly more fluorine in the enamel of sound teeth than in the sound enamel of carious teeth, furnishing the first substantial evidence that an optimum intake of fluorine is related to resistance to dental caries, has received support from two sources. First, field studies have shown a lower incidence of caries in areas of endemic mottled enamel (Arnim, Aberle and Pitney, '37; Klein and Palmer, '38; Dean, '38; Day, '40) and in regions in which the fluorine content of the drinking water is high irrespective of whether or not the teeth are mottled (Dean, Jay, Arnold, McClure and Elvove, '39). Second, the teeth of rats have been found to develop fewer caries when fluorine is added to the diet either during the time of development of the teeth (Cox, Matuschak, Dixon, Dodds and Walker, '39) or after the teeth are mature (Miller, '39; Hodge and Finn, '39).

The mechanism by which fluorine inhibits caries appears to be one of the following, operating alone or in conjunction with the other: (a) an optimum amount of fluorine in enamel confers properties upon it which make it more resistant to caries, (b) the presence of fluoride in the fluids of the oral cavity alters bacterial metabolism and inhibits enzyme action. The first is supported by the analyses of enamel of caries resistant

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teeth (Armstrong, '37; Armstrong and Brekhus, '38 b); by the observation (Volker, '39) that fluorine incorporated in dental tissues reduces their acid solubility; by the continuing protection against caries afforded to the teeth of rats when they were fed extra fluorine only during the time of tooth development (Cox, Matuschak, Dixon, Dodds and Walker, '39); and by the interpretation placed upon the caries inhibiting action of fluoride occurring in rats deprived of salivary glands (Cheyne, '40). The second possibility was offered by Miller ('39), and Dean et al. ('39) tacitly assumed that this is the principal mechanism, but (a) cannot be excluded by the observations of the latter group of workers. The findings of Bibby and Van Kesteren ('39) with regard to the lessened acid production by oral bacteria in the presence of fluorine or fluorosed enamel and dentin may be interpreted in the light of either (a) or (b).

It has been proposed directly (Cox, Matuschak, Dixon, Dodds and Walker, '39) and by implication (Dean, Jay, Arnold, McClure and Elvove, '39) that fluorine be added to communal water supplies having a low content of this element as a means of reducing dental caries in the human. In this connection it would be of value to determine if fluorine can be incorporated into enamel other than at the time of the formation of the tooth. The possible avenues by which fluorine might enter the enamel of erupted teeth are: (a) by diffusion from the blood via the dentin and (b) by direct adsorption and incorporation into the mineral phase from fluids in contact with the tooth surface. Should (b) be feasible the fluorine content of enamel could be increased, when required, by topical application of soluble fluorides to the teeth. This method previously suggested by others (Volker, Hodge, Wilson and Van Voorhis, '40) would greatly reduce the dangers attendant upon the ingestion of excess fluorine and would eliminate the possibility of producing mottled enamel. While the present study was in progress it was shown (Volker, Hodge, Wilson and Van Voorhis, '40) in work also directed at the determination of the feasibility of (b), and in which the radioactive

isotope was used as an indicator, that fluorine is adsorbed from solution by powdered enamel in accordance with the requirements of the Freundlich adsorption isotherm. Since the powdered enamel used in this work possessed a greatly increased surface in comparison to that of a tooth in situ and possibly had altered properties the application of the results to in vivo conditions is uncertain.

The results to be reported will demonstrate that the fluorine content of enamel of molar teeth of mature living rats can be appreciably increased by fluorine which enters it by way of the surface of the teeth.

<div align="center">EXPERIMENTAL</div>

Male albino rats which had been raised to maturity (average weight 250 gm.) on the same stock diet were fed for 60 days on commercial dog biscuits [2] and water, both supplied ad libitum. The food consumption was determined and the water intake measured at regular intervals. The water supplied over the 60-day period to the animals of the experimental group contained 20 p.p.m. of fluorine added as sodium fluoride and that consumed by the control animals was found, by analysis, to contain only a trace of this element. The animals of the experimental group were given, for 5 days before they were killed, distilled water in order to cleanse the crevices and sulci of their teeth of any fluorine which might have been occluded therein. After sacrifice of the animals the femurs and jaws were dissected out and boiled in distilled water until the soft tissues could easily be removed. The teeth, after removal from the jaws, were cleaned with the aid of dental instruments until inspection under magnification showed them to be free of soft tissue, bone, and debris. All specimens were stored in alcohol.

Inspection of the incisors of the experimental group revealed striations of alternating orange and lighter orange or white bands only discernible under magnification and more pronounced on the lower teeth. This effect of excess fluorine ingestion on the growing incisors of rats has been previously

[2] Purina Dog Chow.

described by several observers (McClure, '39) and is noted here only as evidence that the experimental animals received and absorbed a considerably increased amount of fluorine. The incisor teeth of the control animals were entirely normal in appearance.

TABLE 1

Fluorine intake of rats and fluorine contents of enamel, dentin and bone

ANIMAL NO.[1]	FLUORINE INTAKE FROM WATER	FLUORINE CONTENT				
		Incisor teeth		Molar teeth		Dry fat-free femur
		Dentin	Enamel	Dentin	Pooled enamel[2]	
	mg.	%	%	%	%	%
1C		0.0164	0.0190	0.0240		0.0248
2C		0.0153	0.0194	0.0250		0.0199
3C		0.0152	0.0181	0.0240		0.0288
4C		0.0135	0.0140	0.0236	(0.0169)	0.0189
5C		0.0161	0.0186	0.0235	(0.0176)	0.0224
6C		0.0149	0.0157	0.0240	(0.0177)	0.0198
7C		0.0171	0.0186	0.0233		0.0224
8C		0.0147	0.0194	0.0233		0.0186
9C		0.0154	0.0184	0.0243		0.0185
Average		0.0154	0.0179	0.0238	0.0174	0.0215
1E	18.4	0.0340	0.0367	0.0244		0.0484
2E	24.5	0.0270	0.0230	0.0232		0.0476
3E	30.6	0.0300	0.0302	0.0236		0.0375
4E	37.3	0.0343	0.0341	0.0240	(0.0244)	0.0575
5E	36.4	0.0241	0.0261	0.0259	(0.0233)	0.0410
6E	33.6	0.0361	0.0366	0.0245	(0.0236)	0.0574
7E	36.4	0.0394	0.0329	0.0254		0.0508
8E	38.0	0.0384	0.0338	0.0247		0.0428
9E	28.5	0.0251	0.0256	0.0251		0.0353
10E	26.3	0.0250	0.0285	0.0240		0.0484
Average		0.0313	0.0309	0.0246	0.0237	0.0466

[1] The letters C and E indicate animals of control and experimental groups.
[2] See text.

The molar teeth of each animal were pooled in separate lots and the incisor teeth were similarly combined in individual groups. Each lot was separately prepared for analysis (Armstrong and Brekhus, '37) and the enamel and dentin separated (Manley and Hodge, '39) by methods previously described. The bones were broken into bits, wrapped in cloth packets, extracted for 24 hours with alcohol in a Soxhlet apparatus,

dried, and pulverized. The analyses for fluorine were made by the method of Armstrong ('36) with certain modifications, the most important of which were the accurate measurement of the volume of indicator (sodium alizarine sulphonate) and the use of a fluorescent daylight lamp during the titrations.

The amount of enamel obtained from the pooled molar teeth of a single animal being so small (8 to 10 mg.), it was considered that analyses of the required accuracy of the molar enamel of individual animals would not be possible. Accordingly, equal weights of molar enamel from each animal were pooled and mixed, that from the experimental animals going into one sample and that from the controls into a second. The average of the analyses, performed in triplicate, of the pooled molar enamel of each group was the same as would have been obtained had the material from the individual animals been analyzed.

The fluorine intake of the rats during the experimental period and the results of analysis of their femurs, enamel, and dentin are shown in table 1. Sixteen samples of dentin and six of bone were analyzed twice. The average difference of the duplicate analyses was 2.8% and the maximum difference was 11.9%. Ten analyses for fluorine were made on a sample of pooled human enamel at various times during the course of this study. The average difference of the repeated analyses from the mean (0.0114% fluorine) was 2.6%.

<center>DISCUSSION</center>

The molar teeth of the control animals like the permanent teeth of the human (Armstrong and Brekhus, '38 a), with which they are comparable in development, were found to contain more fluorine in the dentin than in the enamel. The higher fluorine (Armstrong and Brekhus, '38 a) and magnesium (Armstrong and Brekhus, '37) contents of dentin of human teeth had been explained by the assumption that dentin is able, in contrast to enamel, to acquire these elements from the blood after the tooth is erupted. Further support for this hypothesis was obtained (Hevesy and Armstrong, '40) when it was shown

that radioactive phosphorus, subcutaneously administered to adult cats, could be demonstrated in appreciable quantities in the dentin but only in traces in the enamel. Since in the present study the molar dentin of the animals ingesting excess fluorine was not found to contain significantly more of this element than the molar dentin of the control animals, it is obvious that some revision is required of the hypothesis relating to the mechanism of interchange between dentin of mature teeth and the blood. With respect to fluorine it is evident, if such a mechanism exists, that it does not continue to act throughout the entire life of the tooth. Possibly the amount of fluorine found in molar dentin (of the order of 0.024%) represents its saturation content with respect to this element. Thus a secondary enrichment could occur until this amount is reached after which time no more fluorine can be deposited in the dentin of teeth in situ. That a secondary fluorine enrichment of teeth like rat molars which remain anatomically unaltered after eruption does occur at some stage subsequent to calcification is rendered probable by a consideration of the results of analysis of the enamel and dentin of the incisor teeth of the control animals. The incisor teeth grow continuously and therefore such teeth are newly formed and have had only a relatively short time to acquire extra fluorine. The fluorine contents of their enamel and dentin would be expected to be very nearly those present at the time of formation of the teeth and to represent, in general, the fluorine contents of recently deposited enamel and dentin. It will be noted that the fluorine contents of the enamel and dentin of the incisors of the control animals are about in the same proportion as the amount of mineral material in enamel and dentin and that the relationship of fluorine contents of the two fractions of these teeth is opposite to that of the molar teeth of the same animals. In consequence of the above considerations these results are evidence that enamel and dentin are not formed with a higher content of fluorine in the latter. Although there is no evidence to support the assumption, it is possible that conditions with respect to developing molar teeth of rats are different from

those pertaining to incisor teeth. If such were the case molar dentin might, at the time of formation, acquire more fluorine than enamel or incisor dentin.

Indirect evidence (Armstrong and Brekhus, '37), supported by direct experiment using radioactive phosphorus as an indicator (Hevesy and Armstrong, '40), has been interpreted to indicate that the composition of enamel of erupted mature teeth is incapable of alteration. However, the mean fluorine content of the molar enamel of the animals which received water containing 20 p.p.m. of fluorine was found to be significantly higher (36%) than that of the control animals. This difference is not due to contamination of the enamel fraction with dentin resulting from imperfect separation since the molar dentin of both groups of animals contained amounts of fluorine which were not significantly different. The latter fact is also evidence that the extra fluorine present in the molar enamel of the experimental animals did not enter it via the dentin. That it could have reached the enamel through the dentin without the fluorine content of the latter being increased would seem most unlikely. It is thus highly probable that the fluorine gained by the molar enamel of the test animals was absorbed through the surface of the enamel exposed to the oral cavity.

While the possibility cannot be excluded it seems certain that the fluorine which entered the molar enamel was derived from the drinking water as it was in contact with the teeth, a process which could continue until dilution with saliva greatly reduced the fluorine content of the oral fluids, rather than from fluorine secreted in the saliva. The relatively enormous amount of bone is known, from the work of numerous investigators, to absorb fluorine effectively from the blood and to retain it—a further example of which is seen from the results of analysis of the bones reported in table 1. Phillips, Hart and Bohstedt ('34) found it difficult to influence the fluorine content of another secretion, milk, by feeding proportionally larger amounts of fluorine than those received by the animals used in this study. In any event the possible increase of fluorine in the

saliva would not alter the conclusion that the extra fluorine entered the molar enamel from fluids in contact with the teeth.

It would appear that the very considerably increased fluorine contents of both fractions of the experimental incisor teeth were due, not only to the increased amount of fluorine received by the animals during the formation of the teeth, but also to adsorption and combination of this element through the surface of the enamel and dentin. The structure of dentin, which in the rat incisor is exposed on the posterior surface of the tooth, would likely facilitate the penetration of dissolved materials into it. On this basis one can account for the fact that the fluorine content of the incisor dentin of the test animals was increased from less than that of the enamel to a value equal to the latter even though the amount of fluorine in the enamel was considerably elevated.

If the findings of this study, insofar as they pertain to the molar teeth, are applicable to the teeth of the human it would appear possible to increase the fluorine content of at least the surface layer of enamel of human teeth by topical application of soluble fluorides. There is every reason to conclude, until positive evidence to the contrary is obtained, that fluorine once combined in enamel structure would remain there in large part. The fact that a high fluorine content of the enamel of the test animals was maintained for 5 days during which the animals consumed distilled water is evidence that the union is not a loose one. Fluorine present in the mineral phase of calcified tissues substitutes for hydroxyl in the structure of hydroxy-apatite (Gruner, McConnell and Armstrong, '37). That the combination of fluorine in the fluor-apatite structure as it exists in enamel is stable is shown by the fact that normal human enamel contains (Armstrong and Brekhus, '38 a) 111 p.p.m. of fluorine, an amount which is far in excess of the fluorine content of any potable water or of the amount of fluorine which could reasonably be expected to occur in saliva or blood. An extreme example of this sort is seen in the case of a woman whose teeth had become mottled as a result of residing, during childhood, in a region of endemic mottled

enamel. Even though she had used Minneapolis city water for 22 years before the teeth were extracted the fluorine content (250 to 360 p.p.m.) of the enamel was found (Armstrong and Brekhus, '38 a) to be much higher than normal. The fact that powdered bone has been found an effective agent for the removal of fluorine from water (Smith and Davey, '39) is further evidence that the mineral salt of calcified tissues can acquire and retain fluorine against a concentration gradient.

SUMMARY

The molar teeth of rats fed an ordinary diet contain more fluorine in the dentin than in the enamel but the reverse obtains with respect to the incisor teeth.

The fluorine content of the dentin of molar teeth of mature rats is not increased when the animals are given water containing 20 p.p.m. of fluorine.

The above facts show that a secondary enrichment of dentin with fluorine occurs after its calcification and that the process does not continue indefinitely.

The fluorine content of the enamel of mature erupted teeth (rat molars) is increased by fluorine which enters it through the surface when rats are given drinking water containing 20 p.p.m. of this element.

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NUTRITIONAL REQUIREMENTS DURING THE LATTER HALF OF LIFE [1]

CLIVE M. McCAY, L. A. MAYNARD, GLADYS SPERLING AND
HARLOW S. OSGOOD [2]

Animal Nutrition Laboratory, Cornell University, Ithaca, N. Y.

ONE FIGURE

Few attempts have been made to determine the optimum diet for the adult after he has attained middle age. Such research is beset with difficulties foreign to similar studies for the first half of life. After the attainment of middle age the body becomes more and more subject to the diseases resulting from the regime followed during the first half. Furthermore, the diseases that accompany ageing appear. These diseases induce strains on the physiological mechanisms and make the evaluation of such factors as dietary variables very difficult. Nevertheless, the importance of the diet during the latter half of life must be of nearly the same order as during the first half. Therefore, it must be studied in spite of the handicaps imposed by the ageing process and in relation to these very changes and diseases which accompany ageing.

In the present studies the white rat has been employed because its life span is short and its diet during early life is well defined. The greatest limitation of the white rat and one which is little appreciated by most workers is the susceptibility, early in life, to diseases of the lungs. This is merely stated here but will be discussed elsewhere in our reports concerning the pathological aspects of our study.

The effect of 8 and 30% protein levels upon longevity

We sought to determine whether the amount of protein modified the length of life when this was introduced as a variable in the middle of the life span. For this purpose, rats were kept until middle age upon our regular stock diet (Maynard, '30). When they had attained a mean age of 485 days, twenty-four were divided into two groups for blood studies. One was fed a diet having a percentage composition of casein 4, salt mixture 4, lactalbumin 3, butterfat 5, cellulose 2, sucrose 10, yeast 5 and cooked starch 67 while the other was fed the same diet with 42% starch and 29% casein.

Blood samples were taken from the hearts of these rats at the start and after they were on the diets for 105 and 187 days. At 105 days there was no significant difference in the value for the non-protein nitrogen level [3] and the means fell between 26 and 32 mg./100 cc. At the 187-day period, however, the N.P.N. had risen to 48 mg. for the rats fed the higher per cent of protein and remained at the previous level for those upon the 8% diet. This experiment was then repeated with rats of similar age. This time the protein level was not reflected in the N.P.N. at the end of 55 days, but a significantly higher level was found for the higher protein group at the end of 112 days.

A second study was run at the same time with the same diet to determine the effect of the protein level upon the life span. The mean length of life for the twenty-two males upon the lower protein diet was 679 ± 17 days and 641 ± 17 days for a similar number upon the higher level. These differences were not significant by statistical analysis.

The effect of 8, 14 and 20% protein levels

A third study was now undertaken using diets similar in composition to those of the first except that carotene was fed as an additional supplement to all groups. Diets containing

[3] The method was that of P. B. Hawk and O. Bergeim: Practical Physiological Chemistry, 9th ed., p. 368.

8, 14 and 20% of protein were used, shifting the casein level as previously. The mean age of the animals at the start was 445 days.

The results in terms of life span are summarized in table 1.

These data indicated no relation between the level of the protein and the length of life when tested statistically, with the exception of females fed the 8% level. These animals showed increased length of life.

Non-protein nitrogen was determined in the blood of eight rats from each group after they had been fed the diet for 116 and 238 days. At both periods the mean values were 30 to 34 mg./100 cc. for those fed the 8 and 14% protein levels but

TABLE 1

Mean life span and level of dietary protein in relation to the weight of ventricle and kidney

PRO-TEIN LEVEL	NUMBER OF ANIMALS		AGE IN DAYS AT DEATH		WEIGHT OF ONE KIDNEY		WEIGHT OF VENTRICLE	
	Males	Females	Males	Females	Male	Female	Male	Female
%					gm.	gm.	gm.	gm.
8	16	30	667	841	1.09(11)[1]	0.93(28)	1.25(11)	1.23(22)
14	15	30	640	769	1.32(14)	1.12(22)	1.57(11)	1.38(21)
20	18	35	654	770	1.32(12)	1.06(26)	1.63(11)	1.43(23)

[1] Values in parentheses show the number of organs weighed.

40 and 41 mg. for those allowed the 20% level. Many determinations of the daily excretion of albumin were made. Upon the highest protein level the values varied from 3 to 273 mg. However, the mean value for the 20% protein level was 82 ± 15 mg.; that for the two lower levels was 23 ± 6. Earlier attempts to correlate this excretion of albumin by the rat with the protein level of the diet had failed (McCay and Nelson, '26).

When animals died and were found while the tissues were fresh, weights of organs were obtained. The means of these values are assembled in table 1 for the ventricles and kidney weights. The weights of these organs in both sexes showed increases correlated with increased protein intake.

Fourth study using 8 and 20% protein

In the fourth study more attention was given to the albumin of the urine. This experiment was similar to the preceding ones and can be summarized in table 2.

Fifth study of protein level and such modifying factors as quality of the protein, exercise of the animals and restriction of body fatness

For this study two groups of male rats were kept until middle age upon the stock ration. At the time of starting the diets one group was 200 to 300 days of age and the other 400 to 450. After discarding all animals that did not appear in good health, 329 rats remained. These were divided into sixteen groups as evenly as possible from a consideration of body

TABLE 2

Level of protein and weight of organs

LEVEL OF PRO-TEIN	MEAN LIFE SPAN		MEAN KIDNEY WEIGHT		MEAN VENTRICLE WEIGHT		MEAN BODY WEIGHT	
	Male	Female	Male	Female	Male	Female	Male	Female
%	*days*		*gm.*		*gm.*		*gm.*	
8	673(22)	839(21)	1.20	1.02	1.25	1.23	265	194
20	716(22)	818(20)	1.75	1.14	1.54	1.44	272	192

weight, age and litter of origin. Thus each group contained either twenty or twenty-one individuals.

In order to get a better idea of the development of pathological changes before death, four from each of the sixteen groups were killed at intervals in the course of the study. The remainder of sixteen or seventeen rats in each group were kept until death, as in earlier studies.

The regimes for each of the sixteen groups are shown in table 4. These rats were housed in an air-conditioned room with the temperature maintained as nearly as possible at 78°F. and the humidity at 40 to 50%. All natural light was excluded from the room.

Throughout this study the short term "casein" is used to designate those groups receiving a mixture of lactalbumin and

casein as the major source of protein. The terms "thin" and "fat" are used to describe groups restricted and non-restricted, respectively, in their daily allowance of calories.

The purposes of these studies were (1) to compare the effects of high and low protein diets when consumed during the latter half of life, (2) to compare casein and liver as protein sources at two levels, (3) to determine the effect of forced exercise versus normal activity and (4) to compare animals allowed to

Fig. 1   Barrels equipped with a sewing machine belt running in bicycle rims and attached to a small motor and reducing gears, for exercising rats.

fatten with those kept thin in body weight during the latter half of life. The divisions described above made it possible to compare groups of twenty rats or to combine groups into large ones with 164 individuals for comparison. Thus all rats on high protein diets could be compared with all on low protein ones or all rats exercised could be compared with all not exercised.

Exercise was given by forcing the rats to run in rotating barrels shown in figure 1. The rate of rotation of the barrels

was first set very slow. Gradually the rate of rotation of the barrels was increased until the length of exercise period was extended to 2 hours daily. Exercise was started in February, 1937 and continued until November, unless rats became obviously sick. From our experience there is no doubt that middle-aged rats are killed by exercise if they are badly diseased or if they are not given a preliminary period of training.

In groups subjected to body weight restriction, the "thin" ones, the reduction of body weight was made by limiting the food eaten in these groups in order to hold the body about 10% below the maximum weight attained by individuals when

TABLE 3

Percentage composition of diets used in fifth study

| | HIGH PROTEIN | | LOW PROTEIN | |
	Casein	Liver	Casein	Liver
Cooked starch	34	34	65	65
Casein	29	—	4	—
Lactalbumin	4	—	3	—
Sucrose	10	10	10	10
Cellulose	2	2	2	2
Butter	5	5	5	5
Lard	7	—	2	—
Yeast (irradiated)	5	5	5	5
Salt mixture	4	3	4	3
Dry liver	—	41	—	10

eating all the food desired and not exercising. The non-restricted groups were allowed all the food desired.

The four diets used in this study were constituted as shown in table 3. A supplement to this diet given to all rats was 1 mg. of carotene added to each kilogram of feed. In addition, every rat was given four yeast tablets per week. Each tablet weighed a third of a gram and carried 0.02 mg. of carotene.

Restricted and non-restricted groups all received the same amount of basal diet, but the non-restricted or "fat" rats were allowed all they wished of a mixture of sucrose 4, starch 4 and lard 1, in addition to the regular diet.

The salt mixture was prepared by mixing 3280 gm. of bone meal with an equal weight of limestone. To this was then

added 1640 each of KCl and NaCl. To this, in turn, was added 130 gm. of $MgSO_4 \cdot 7 H_2O$ and 30 gm. of ferric citrate. The trace elements, consisting of $ZnSO_4 \cdot 7 H_2O$, 5 gm., $CuSO_4 \cdot 5 H_2O$, 1.6 gm., $MnSO_4 \cdot 5 H_2O$, 10 gm., $AlCl_3 \cdot 6 H_2O$, 5 gm., KI, 0.06 gm., were dissolved and sprinkled over the bone meal.

The rats of all sixteen groups were kept upon the diet until they died, with the exception of four from each group killed for study. Post mortem examinations were made in all possible cases. Reports of these examinations will be made elsewhere. Throughout this study x-ray photographs were used extensively in following calcification changes in cartilages, bone and tissues. These results will be summarized in a later article. All organs were weighed at the time of death, before they were fixed for histological study.

Statistical analyses were made of the data from each of the sixteen groups, as well as of combinations of these groups. All data are summarized in table 4. Conclusions are based upon these data.

RESULTS

Life span

None of the variables discussed in this report modify the length of life in a manner comparable to the retardation of growth discussed previously (McCay et al., '39). Of the sixteen groups, fourteen differ more than enough from others to give significant odds. Four combinations likewise differ to the same extent from four other combinations. In the groups given the higher protein, those fed liver outlived those fed milk proteins. Likewise those restricted in food intake in order to avoid excessive fatness of the body lived longer than those allowed to fatten at will. Restriction was more favorable in those fed liver at a high protein level than in those fed milk proteins.

The group that stands out from the others with respect to length of life is that fed a low protein diet with liver as the source of protein, kept thin and given exercise (13). This combination of factors was undoubtedly the most favorable.

TABLE 4

Diet, life span and organ weights

GROUP NO.	DIET	MEAN LIFE SPAN	P.E.M.[1]	MEAN BODY WEIGHT	P.E.M.	MEAN HEART WEIGHT	P.E.M	MEAN KIDNEY WEIGHT	P.E.M.	MEAN LIVER WEIGHT	P.E.M.	MEAN SPLEEN WEIGHT	P.E.M.	MEAN TESTES WEIGHT	P.E.M.
		days	days	gm.	gm.	gm	gm.	gm.	gm.	gm.	gm	gm.	gm.	gm.	gm.
1	High protein–casein–exercise–thin	614	23	272	7	1.57	0.08	1.62	0.08	11.8	0.9	0.97	0.06	1.36	0.18
2	High protein–casein–exercise–fat	563	16	320	13	1.31	0.05	1.62	0.15	12.3	0.6	0.75	0.05	1.71	0.13
3	High protein–casein–no exercise–thin	544	19	249	7	1.31	0.07	1.30	0.04	10.5	0.7	0.80	0.08	1.39	0.17
4	High protein–casein–no exercise–fat	572	21	311	14	1.31	0.07	1.53	0.08	11.5	0.7	0.87	0.09	1.13	0.09
5	High protein–liver–exercise–thin	663	24	260	8	1.40	0.07	1.57	0.06	11.4	0.6	0.81	0.09	1.24	0.12
6	High protein–liver–exercise–fat	574	17	244	7	1.55	0.09	1.60	0.07	12.8	0.7	0.75	0.07	1.15	0.13
7	High protein–liver–no exercise–thin	677	28	224	5	1.28	0.05	1.42	0.06	10.1	0.7	0.53	0.03	1.00	0.10
8	High protein–liver–no exercise–fat	609	22	240	7	1.32	0.07	1.37	0.07	11.4	0.5	0.53	0.03	1.30	0.14
9	Low protein–casein–exercise–thin	597	31	263	8	1.38	0.07	1.17	0.03	9.2	0.7	0.74	0.07	0.97	0.11
10	Low protein–casein–exercise–fat	590	22	269	10	1.32	0.05	1.23	0.05	10.7	0.7	0.62	0.05	1.26	0.09
11	Low protein–casein–no exercise–thin	641	28	229	8	1.46	0.09	1.19	0.06	8.7	0.5	0.62	0.04	0.95	0.07
12	Low protein–casein–no exercise–fat	581	19	313	11	1.32	0.07	1.29	0.03	11.5	0.9	0.68	0.08	1.86	0.15
13	Low protein–liver–exercise–thin	707	30	239	9	1.25	0.05	1.16	0.03	10.6	0.4	0.69	0.06	1.11	0.12
14	Low protein–liver–exercise–fat	621	28	253	.8	1.19	0.05	1.07	0.03	10.5	0.8	0.85	0.11	1.31	0.12
15	Low protein–liver–no exercise–thin	587	28	257	8	1.25	0.07	1.13	0.13	10.6	0.5	0.49	0.05	1.25	0.13
16	Low protein–liver–no exercise–fat	597	22	243	7	1.29	0.05	1.17	0.09	10.6	0.6	0.65	0.05	1.23	0.11

[1] P.E.M. (probable error of the mean).

Among eight groups, this one was favored by odds ranging from 30:1 to 450:1.

Among the high protein groups, that fed liver and restricted without exercise outlived a similar group given exercise (see 7). In the case of animals exercised on the high liver diet the better group was that restricted.

In table 5 are shown the number alive in each group at intervals of 100 days. The 700-day period is rather a crucial one

TABLE 5

Number of rats alive at age intervals of 100 days and the ages attained by the oldest in each group

GROUP NO.	DIET	NUMBER ALIVE AT 100-DAY INTERVALS						AGE OF THREE OLDEST AT DEATH (DAYS)		
		500	600	700	800	900	1000			
1	High protein–casein–exercise–thin	14	9	4	1	0	0	769	792	889
2	High protein–casein–exercise–fat	11	8	2	0	0	0	681	708	743
3	High protein–casein–no exercise–thin	10	4	3	0	0	0	747	754	781
4	High protein–casein–no exercise–fat	13	6	3	1	0	0	730	738	862
5	High protein–liver–exercise–thin	13	10	7	4	0	0	851	853	871
6	High protein–liver–exercise–fat	12	7	2	0	0	0	652	717	751
7	High protein–liver–no exercise–thin	14	12	8	1	1	1	766	768	1131
8	High protein–liver–no exercise–fat	13	7	4	1	1	0	707	761	977
9	Low protein–casein–exercise–thin	13	9	5	2	1	1	783	832	1009
10	Low protein–casein–exercise–fat	13	9	4	1	0	0	712	732	856
11	Low protein–casein–no exercise–thin	12	11	8	3	1	0	804	839	900
12	Low protein–casein–no exercise–fat	12	9	3	0	0	0	712	772	787
13	Low protein–liver–exercise–thin	14	10	7	6	3	1	947	985	1062
14	Low protein–liver–exercise–fat	11	11	7	1	0	0	791	795	822
15	Low protein–liver–no exercise–thin	9	8	5	2	0	0	743	814	877
16	Low protein–liver–no exercise–fat	14	11	3	1	0	0	730	732	805

in the life of the rat since it corresponds to about 70 years in the life of man. At this time there were forty-seven rats alive in the restricted groups contrasted to twenty-eight in those not restricted. This is more marked for this variable than the 33:42 ratio found for high versus low protein.

There is little evidence to indicate that exercise plays an important role in the latter half of life. Perhaps the rat, being subject to lung disease, is not a good animal for such a study.

Exercise is probably advantageous for the healthy and disastrous for the sick rat.

As a rule the restriction of the calories of the diet is favorable for increasing the span of life, although in two groups this was not true. In general, rats kept underweight outlive those allowed to fatten as they will.

These data, as well as several earlier studies, afford little evidence that the level of protein in the diet affects the life span, if the limits are not too extreme. Of all groups, however, the best was that obliged to exercise, maintained thin, and fed a low protein diet supplied by liver. In most cases liver was more satisfactory as a source of protein than the milk proteins. Possibly this is subject to other interpretation, since the liver may have carried essentials such as unrecognized substances not found in the milk proteins.

As rats live to greater ages the body weight at the time of death becomes progressively less. This is only indirectly related to the diet, inasmuch as the diet modifies the life span. Among the sixteen groups studied, the mean difference between the maximum body weight attained and the weight at death varied from 129 to 194 gm. At the time of death the mean body weights for the different groups varied from 229 to 311 gm. No further conclusions can be drawn from the final weights as they were the result of the terminal disease for the most part.

The maximum body weight and life span

A spot diagram was prepared showing the relation of the maximum body weight attained at any time during life and the life span. The rats that lived the longest were those with intermediate body weights. This favorable zone for maximum life span lay between 350 and 450 gm. No animal whose body weight at any time in life exceeded 450 gm. was able to exceed 810 days for its life span. This was also true for animals whose body weights never exceeded 360 gm. For our colony the favorable zone lies within these limits of 350 to 450 gm. This is shown in the abridged table 6 prepared from the spot diagram.

Extreme age

Table 5 shows the age attained by each of the three oldest in each group. These data give some clue to the factors responsible. In the present study three individuals attained ages exceeding 1000 days. This is unusual among male rats of our colony. Two of these individuals were in the groups (9 and 13) fed low protein with restriction in food consumption and forced to exercise. The oldest three survivors were in such a group (13) with the additional variable of liver as the source of protein. The group also had the highest mean life span. It is, therefore, probable that the conditions under which this group lived were the most favorable, namely, low protein with liver

TABLE 6

Relation between the maximum body weight attained and the life span of rats in per cent of the weight group

MAXIMUM BODY WEIGHT	AGE AT DEATH (DAYS)					
	250–409	410–569	570–729	730–889	890–1049	1049 up
gm.						
Below 330	67	33	0	0	0	0
330 – 389	10	38	30	20	2	0
390 – 449	4	27	44	21	3	1
450 – 509	2	26	54	18	0	0
510 up	15	15	62	8	0	0

furnishing this protein. They also had exercise and were not allowed to become as fat as possible since their food intake was restricted.

For attaining extreme age, liver seems more favorable than the milk proteins. Exercise seems more favorable on a low protein diet than it does on a high one. Prevention of over-fattening of the body seems important at both protein levels.

The weight of the heart

Only a few significant differences are found in the heart weights. The hearts of those fed the high liver diets were significantly heavier than those fed the low liver diet. This difference is also found between the small hearts of those fed a low

level of liver and the large hearts found when milk proteins at a high level with exercise and restriction were the other variables.

The weight of the kidneys

The well-known effect of protein upon the size of the kidneys is found throughout these data. Inasmuch as this is well known (Cowdry, '39) the interesting features to report here are that this enlargement resulted from the level of protein during the latter half of life and that these kidneys reflected the protein level at the death of the animal. The degree of wasting which accompanied the last diseases of old age did not obliterate the enlargement of the kidneys produced by the high protein diet. With one exception all low protein groups were very significantly different from corresponding high ones.

Of the groups fed high protein diets it was found that those which were forced to exercise (groups 1, 2, 5, 6) had heavier kidneys than those not exercised (groups 3, 4, 7, 8), whether they were fat or thin. The increase due to exercise was greatest in the thin animals fed casein—0.32 gm. (groups 1 and 3). Among individual groups in the higher protein series there were six that varied with odds greater than 30 : 1 while there was only one such case in the lower protein series. This suggests that several factors come into play when the kidneys are enlarged as a result of high protein diet. Among these, exercise, body fatness, and source of protein all play a part. Of course, none of these are as marked as the contrast between the small kidneys from the low protein diet and the larger ones from the high.

The weight of the liver

In considering organ weights no attempt has been made to relate the organ weight to the body weight at death inasmuch as groups were adjusted at the beginning of the experiment, in middle age for the rat, so that groups were equal in regard to size and weight of individuals. Relations of body weight and organ weight at the end represent the relative wasting of

the body in old age and the effect of the diseases involved at the end of life.

The livers of the animals fed the higher protein diet were heavier than those fed the lower level. This difference amounted to about 10% in contrast to that of the body weights which differed by about 3%. The restriction in the diet left its reflection in the weight of the liver even when rats died from natural causes. The livers of rats restricted in food intake were lighter in weight as one would expect. This is especially marked in the groups fed low protein diets with milk proteins as the source. The occurrence of small livers in the group fed milk proteins at a low level, without exercise and restricted is probably related to the low body weights at the time of death.

The weight of the spleen

The presence of so many significant variations in the weight of the spleen as a result of these different regimes is surprising. The total of these amounted to twenty-six, probably more than the number found in kidneys. These differences were much more prevalent in the high protein groups than in the low. The spleens of rats fed casein were larger than those fed liver at the higher level. The higher milk protein level produced larger spleens than the lower one. In the case of the higher protein level those groups exercised had larger spleens. Those fed higher levels of milk proteins had larger ones than those fed the higher amount of liver.

Krumbaar (Cowdry, '39) found that the weight of the spleen followed definite trends in relation to age but that there were great variations. This is what might be expected if factors of diet and regime can produce significant differences. The tendency of the spleen to reflect the state of nutrition must be much more marked than anyone has observed previously. Protein must be a very important dietary factor in modifying the spleen since so many significant differences were found for the higher level. The only one within the lower protein level occurred in those fed liver. This difference lay between the exercise variables in the non-restricted groups.

Testes

The significant differences in the weight of the testes are found in groups with levels of protein intake different from those characterizing any other organ differences. In contrast to the spleen the significant variations for the testes are found for the most part in the groups fed low levels of protein. In the lower protein levels those fed restricted intakes had smaller testes with the effect much more marked in the milk protein groups. Those fed the higher levels of casein had larger testes than those fed the lower. Within the groups fed the higher casein level, exercise was the factor producing the larger testes. From the whole picture, protein seems to be the dominant factor in determining the size of the testes at death. At lower levels of protein, the restriction of calories becomes very important.

The lipids of the organs

In three of the four animals killed from each group, the total lipids, the lipid phosphorus and the cholesterol were determined upon the fresh tissues. Two series of significant differences were found. Animals restricted in food intake had lower levels of both cholesterol and total lipids in both the heart and liver, while the lipid phosphorus values were about the same. The livers of animals fed liver diets were richer in all three lipid fractions than those fed casein diets, while the hearts showed no differences. The high and low levels of liver in the diet produced about the same lipid composition in the organs of rats. None of these differences were found in the kidneys.

Comparison of weights of organs of sacrificed rats with those of rats dying of natural causes

To determine if the killing of a small number of healthy rats in the course of a life span experiment affords any clue to the weight of the organs of rats from a given group at the time of death, correlation coefficients were determined between the

weights of organs of those killed and those that died in old age in each group. The values for "r" were the following:

ORGAN	r	P.E.
Liver	0.66	±0.09
Heart	0.005	
Testes	−0.033	
Kidneys	0.63	±0.10
Spleen	0.31	±0.14

From the killing of a few representatives of given groups one can anticipate the weight of certain organs such as the kidneys or liver at the time of natural death. To a limited extent even the weight of the spleen can be foretold roughly. On the other hand, the testes and even the heart must be much affected by the terminal disease since their weights bear little relation at the time of death to the weights of comparable organs from members of the same group killed while in good health.

SUMMARY

Five studies have been completed to determine the effect on rats of different diets and regimes introduced in middle life. The protein level of the diet during the latter half of life does not affect the life span significantly when the protein constitutes 8 to 30% of the diet. At the higher level of protein intake, the non-protein nitrogen of the blood increases and the heart and kidneys become larger. The albumin of the urine varies widely but is roughly correlated with the protein level of the diet. The factors of most importance during the latter half of life, so far as the life span is concerned, are those which determine the degree of fatness of the body. Rats kept underweight by restriction of calories outlive those allowed to fatten. Exercise is of minor importance in comparison with body fatness. The optimum conditions for a long life proved to be thin bodies, exercise, and a low protein diet with this protein provided by liver.

A simple method for exercising animals is described.

The weights of the organs of rats killed while being subjected to different regimes in late life correlate with those of

the rats that die a natural death in the case of the liver and kidneys but not in the case of the heart and testes.

Inasmuch as the major disease that afflicts rats during the latter half of life is one that involves the lungs, it is questionable whether the effects of high protein intake on rats are comparable to those on man.

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SEVERE CALCIUM DEFICIENCY IN GROWING RATS[1]

I. SYMPTOMS AND PATHOLOGY

MURIEL D. D. BOELTER[2] AND DAVID M. GREENBERG

Division of Biochemistry, University of California Medical School, Berkeley

THREE FIGURES

Although the literature concerning the role of calcium in metabolic processes is extensive, no one has reported studies of what might be called uncomplicated calcium deficiency. Most of the dietary regimes previously employed either were not so low in calcium as can now be obtained, or were not carried along for a sufficient period of time to exhaust the animal's calcium supply. It is the purpose of the present paper to report studies in which these objections have been met. This study was undertaken as a sequel to the investigation of the effects of magnesium deficiency carried out by Tufts and Greenberg ('38).

EXPERIMENTAL METHODS

Diet. The composition of the basal diet and of the control and low calcium salt mixtures has been published in a previous paper (Kleiber, Boelter and Greenberg, '40).* The diets employed in this study were similar in composition to those used by Tufts and Greenberg ('38) in their work on magnesium deficiency. The salt mixtures were prepared so as to contain

[1] Aided by grants from The Rockefeller Foundation and The Christine Breon Fund for Medical Research. Technical assistance was furnished by the personnel of W.P.A., official project no. 65-1-08-62. A preliminary communication has been previously published (Greenberg, Boelter and Knopf, '39).

[2] The material of this paper was taken from a thesis submitted by Muriel D. D. Boelter to the Graduate Division in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May, 1940.

* Table 1 in the paper of Kleiber, Boelter and Greenberg ('40) giving the basal diet and salt mixtures used in this study, contains two errors. The values of NaCl in the Ca-deficient and control diets are transposed, and the dosage of the crystalline vitamin supplements is per kilo of diet.

a minimum number of constituents in order to facilitate their purification. All salts used were recrystallized to free them from contaminating substances as far as was feasible. The rice bran extract,[3] used as a supplement to supply certain members of the B group of vitamins, was re-extracted with 50% alcohol.

Analysis of the diets showed that the control contained 546 mg. Ca, 84 mg. Mg, and 436 mg. P, while the usual calcium-low food contained 9.4 mg. Ca, 85 mg. Mg, and 425 mg. P per 100 gm. food. Low calcium diets containing about 4 and 20 mg. Ca per 100 gm. were also employed in certain of the experiments. The diet with only 4 mg. of Ca per 100 gm. food was obtained by greater care in purifying the individual components.

The selection and care of the rats and the methods used for the analysis of blood, tissue, and body constituents were the same as those employed by Tufts and Greenberg ('38).

<div align="center">SYMPTOMS AND SEQUELAE</div>

<div align="center">

1. Growth
</div>

The first noticeable effect of the calcium deficient diet is upon the growth rate. In general, there is no other noticeable manifestation of the deprivation for about 2 months. When the animals are allowed to feed ad libitum, the difference between the weight gained by the deficient and the control animals becomes pronounced at the end of the second or third week. At this time the controls gain over 20 gm. per rat per week, whereas the corresponding gain of the deficient animals is 10 gm. or even less. After 4 or 5 weeks, the deficient animals usually cease to grow, and, in fact, may even decline in weight. These facts are illustrated in the growth curves given in figure 1.

The decreased rate of growth of the calcium-deficient rats is, to a large extent, the result of decreased appetite. The average food consumption of rats fed the deficient diet is

[3] Vitab B.

6 to 7 gm. per rat per day as against about 12 gm. for the controls. The weight of the calcium-depleted animals is also adversely affected by the fact that their metabolic rate is higher than that of the controls (Kleiber, Boelter and Greenberg, '40).

Within limits, the time required for the appearance of the symptoms of calcium deprivation, including the retardation
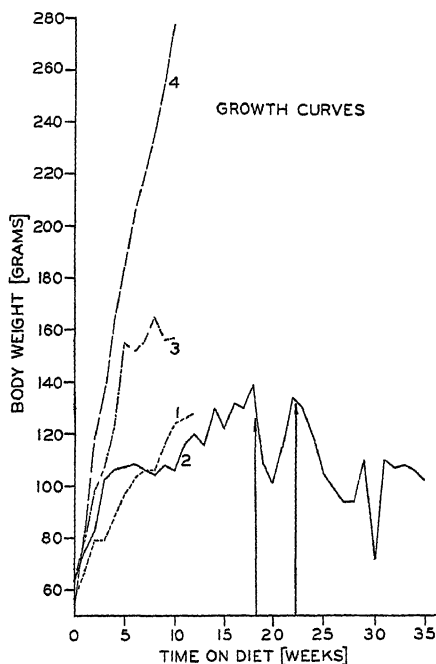

Fig. 1   Effect of the calcium level of the diet on the growth of rats. Average starting weights varied from 55 to 63 gm. Curves are drawn from data of groups of six or more animals. The arrows indicate the time of onset of paralysis in several animals of the group. Curve 1, about 4 mg. Ca per 100 gm. diet; curve 2, about 10 mg. Ca per 100 gm. diet; curve 3, about 20 mg. Ca per 100 gm. diet; curve 4, control, about 550 mg. Ca per 100 gm. diet.

of the growth rate, varies with the calcium content of the diet, taking longest with the diet containing 20 mg. Ca and least with the 4 mg. Ca per 100 gm. food (fig. 1).

Between the weights of about 40 to 100 gm., the size of the animals at the time they are placed on the synthetic low cal-

cium diet merely affects the ultimate weight which the rats attain. The similarity between the growth curves of rats placed on the deficient diet at ages of between 25 to 42 days, respectively, is shown in figure 2. The larger animals maintain
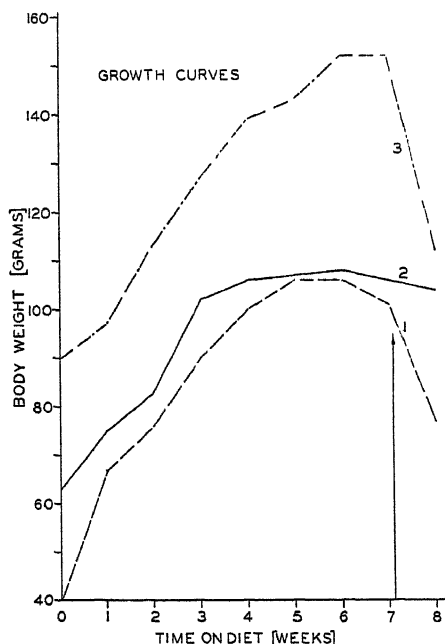

Fig. 2   Influence on growth of weight of animal at beginning of calcium deprivation. Calcium content of the deficient diet is about 10 mg. per 100 gm. diet. The curves are drawn from data of groups of six animals each. The arrow indicates the time of onset of paralysis of the hind quarters of some animals in each group. The average weight and starting age of the animals were: curve 1, 40 gm., 25 days; curve 2, 63 gm., 32 days; curve 3, 90 gm., 42 days.

their advantage in weight throughout the experimental period, but suffer the same consequences at approximately the same time as the younger group. This is indicated by the arrow in figure 2.

2. Symptoms of calcium deficiency

The calcium-deficient animals usually show no outstanding characteristics different from the normal for from 7 to 10

weeks, and some show no ill effects for the whole of their survival time.

In the first place, the animals show a decreased sensitivity and reactivity. They do not exhibit the usual curiosity shown by the control rats, but are inert and quiescent, moving around the cage very little, and evidencing no interest in the proceedings around them. *In no instance was tetany induced in the rat by lack of calcium in the diet alone.* Such potent stimuli as the galvanic current or the sound of an air blast failed to elicit the convulsive seizures of tetany.

After 6 to 9 weeks, there may be a loss of hair, varying from a slight thinning of the coat to a baldness extending over the whole of the dorsal surface of the animal. The possibility exists that the loss of hair may be the result of a disfunction of the vitamins of the B [4] group, since administration of a high level of riboflavin during the first few weeks on the deficient diet favors the maintenance of a healthy coat and may altogether prevent the appearance of baldness.

After about 2 months or more, any one or all of the more drastic symptoms may appear. These include prostration, paralysis of the hind extremities, black feces, and hemorrhages from the anus and from the toe-nail bed. Only a relatively small percentage of the animals succumb spontaneously to prostration and paralysis. The incidence is recorded in table 1.

The paralytic foot-drop of one or both hind legs is most probably the secondary effect of hemorrhages in the central nervous system. The paralysis varies in severity depending upon the degree of calcium depletion of the rat. In a severe case of paralysis, the hind legs are outstretched backward or to one side. If the animal is not entirely helpless, but can move a little, it drags the hind limbs, pushing itself forward with its fore limbs. Immediately after an animal becomes

[4] That the lack of calcium, and not an inadequacy of vitamins, is the ultimate cause of the observed deficient syndrome is proved by the fact that animals maintained on a restricted control ration, so that they actually consume significantly less food than the deficient rats, show none of the symptoms attributed to calcium lack, but are normal in every respect except rate of growth.

paralyzed, it is extremely sensitive to touch and squeals continuously even if handled gently.

Sometimes the rat may be completely prostrated for a week, and, being unable to consume any food, may consequently suffer a great loss of weight. This is illustrated by curve 2 in figure 1. The time of onset of paralysis is indicated by the arrow.

TABLE 1

Incidence of death and paralysis on low calcium diet of a total of seventy-eight rats

TIME ON DIET IN WEEKS	DEATHS			SPONTANEOUS PARALYSIS		
	Number[1]	% of total	Summation of %	Number	% of total	Summation of %
3	2	2.6	2.6			
4	1	1.3	3.9	·1	1.3	1.3
8	5	6.4	10.3	5	6.4	7.7
9	1	1.3	11.6	2	2.6	10.3
10	3	3.9	15.5	1	1.3	11.6
11	11	14.0	29.5	2	2.6	14.2
12	4	5.1	34.6	1	1.3	15.5
13	6	7.7	42.3	1	1.3	16.8
14	2	2.6	44.9	1	1.3	18.1
15	0	0	44.9	2	2.6	20.7
17	4	5.1	50.0	1	1.3	22.0
18	1	1.3	51.3	1	1.3	23.3
19	2	2.6	53.9			
20–23	5	6.4	60.3			

[1] Animals that did not die were used for experimental purposes after being 12 to 20 weeks on the diet.

More than half of the animals suffering spontaneous paralysis will succumb within 24 hours. Of those surviving the initial shock, the majority recover in 2 to 3 weeks, after and during which time they are, of course, liable to repeated attacks. Except for the animals that die of inanition resulting from prolonged prostration, death appears to be due to shock.

Frequently, the skull of a dead rat in a cage will be gnawed through and the brain eaten by the surviving animals.[5] No

[5] Magnesium deficient rats show a preference for eating entrails of deceased cage mates, although often every part of a dead animal will be devoured but the skin, which, found in the morning in a neatly rolled up bundle, may be the only remaining evidence of the cannibalistic feast.

other part of the carcass has ever been attacked, and there is no evidence to indicate that a prostrated animal is ever attacked before its decease.

The neurological condition and the poorly calcified bones, which do not provide adequate skeletal support, affect the posture and movements of the animals in various ways. The rats show an unsteady gait, walking with a waddling movement or hopping in order to protect either one or both hind legs. They have difficulty in standing erect, and usually squat on their haunches with the hind legs tucked under. They may walk stiff-legged, putting down each foot with great care. Sometimes they push themselves backward or in a circle, using the fore limbs only and pivoting on the hind quarters. Conditions similar to the above are passed through on recovery from a case of severe paralysis, or they may result from mild spontaneous attacks not actually involving paralysis of the hind limbs.

The bones of the calcium deficient animals are easily broken, and spontaneous fractures of the leg bones are common.

3. Effect of electrical stimulation

The prostration and paralysis may be induced at a much earlier date (4 weeks) than they occur spontaneously by means of mild, short galvanic shocks from an induction coil, and there is 100% response to this stimulus after 6 to 7 weeks on the calcium-low diet. The electrical stimulus causes an immediate collapse and the subsequent paralysis of the calcium-deprived rat. The animal falls on its back or rolls over on its side, the head and hind limbs being drawn together with the latter flexed against the abdomen. Even if it is not completely prostrated, it shows little ability to right itself or to grasp objects with its paws. The animal is not unconscious as in tetany, but it responds poorly and sluggishly to stimuli. As in spontaneous cases, the animal is extremely sensitive to touch immediately after it has recovered from the initial shock, but within 24 hours the surface of the body loses sensibility and the animal no longer shows any response to even painful

superficial stimuli. The fore limbs of the rat are not involved in the reaction to the electric shock, but remain relatively normal throughout. Severely prostrated animals are incontinent. The appearance of an animal suffering from a severe case of paralysis is illustrated in figure 3.

The amount of current sent through the animal is extremely small and of brief duration. The deficient rats are affected practically instantaneously, whereas a normal animal is merely annoyed and irritated by even prolonged electrical stimulation. The stimulus of the sound of an air blast, which is so
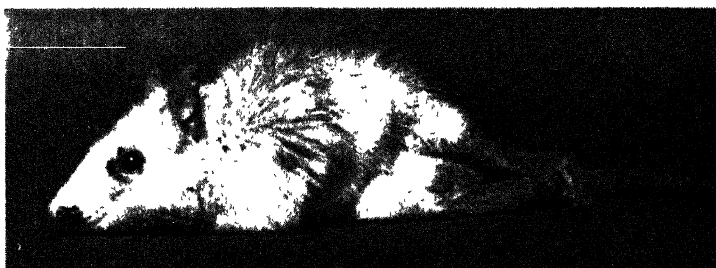

Fig. 3   A calcium deficient rat photographed when it had been 9 weeks on the deprivation regime, 6 days after the induction of paralysis by galvanic stimulation. Note the helpless paralytic condition of the hind limbs, the encrusted nares, the size, and complete prostration of the rat. This animal died 2 days later, weighing 82 gm. Hemorrhagic areas were observed in the lungs, brain, liver, intestines, and gluteal muscle.

effective in causing tetanic convulsions in animals reared on a low magnesium diet, does not disturb the calcium-deprived rats at all.

Calcium-deficient rats that have survived a convulsive dose of strychnine also develop a paralysis of the hind quarters.

4. Longevity

The span of life is greatly reduced and the mortality rate is greatly increased in the animals on the low calcium diet. In a series of observations on seventy-eight animals, 60% died in from 3 to 23 weeks, while, in the same period, not a single death occurred among the animals on the control diet. The mortality statistics and the incidence of the onset of spontaneous seizures of prostration and paralysis are given in table 1.

5. Recovery

Animals that survive the shock of either a spontaneous or electrically induced paralytic condition may recover completely, probably due to resorption of the hemorrhagic clot, even though they continue to ingest the calcium-low food. Recovery is hastened, however, if the animals are transferred to the normal control diet. In some instances there is a permanent loss of the use of one of the limbs even though the animal recovers in every other respect. These animals drag the affected limb along in much the same manner as is sometimes observed in human cases surviving a hemoplegia.

Four weeks after the control level of calcium is added to the rations of the deficient rats, they are no longer susceptible to the induction of paralysis by the electric shock. This was determined on animals that had been on the low calcium regime for from 10 to 14 weeks, and had suffered either spontaneous or induced paralysis and hemorrhages before they were placed on control food. For the first few weeks on the recovery diet, they react as usual, the effect of the galvanic current decreasing as the time on the normal diet progresses. Immediately after the rats are transferred to the control diet, the amount of food consumed returns to the normal level and likewise the weight gain increases to 20 to 30 gm. per rat per week. The animals were found to have a normal blood calcium concentration after 7 weeks on the calcium-adequate diet. Eventually it is impossible to distinguish such rats from normal animals in appearance and reactions.

6. Effect of injection of calcium salts

On the other hand, injections of calcium salts do not protect the depleted animals from the consequences of the galvanic shock and coincident paralysis produced by it. In fact, invariably the calcium injection causes the death of the rat within 24 hours, even if the animal is not subjected to a galvanic shock. The outstanding autopsy finding to which the cause of death can be ascribed is the rupture of the ventricle of the heart in a longitudinal line from the base to the apex. In the calcium low animals, the ventricular wall is abnormally thin,

and the added calcium ion in the circulation possibly causes the heart to stop in systole, and engorge the ventricle with blood, so that it bursts. The lungs, auricles, and all of the blood vessels of, and those leading to, the heart are engorged. Most of the animals die within 3 to 5 hours after the injection.

Similar results were obtained with doses of 5 mg. of calcium in the form of the chloride, lactate, and gluconate, per 100 gm. of body weight. Eleven out of fourteen calcium-deficient animals, and not a single one of the controls, died following injection of the calcium salts. Twice this dosage of calcium had no effect on the corresponding control rats.

Hoff, Smith and Winkler ('39) have recently reported that calcium chloride caused the death by circulatory failure of normal animals within a few hours after its injection. The salt produced fibrillation of the heart before death, and autopsy revealed a dilated heart and engorged pericardium. These authors state that calcium has a direct depressant effect on the heart muscle.

7. Autopsy and histological findings

The conspicuous feature found on examination of the calcium-deprived animals after death from shock and paralysis is the hemorrhage which may occur in practically every internal organ of the body. Massive hemorrhagic areas are frequently noted in the muscles of the gluteal region. The liver and the auricles of the heart, or both, may be engorged with blood, and not infrequently the latter may rupture. Hemorrhages have been found in the lungs, brain, bladder, bone, gastrointestinal walls, nail matrix, and in the peritoneal cavity. The intestinal contents are usually very dark, and frequently the rats have diarrhea. The intestinal hemorrhage appears to originate in the duodenum, as it is at this point that the intestines become black.

The skull is practically uncalcified and paper thin. The rest of the skeleton also is very poorly calcified. It has already been pointed out by Kleiber, Boelter and Greenberg ('40) that calcium-deficient rats are shorter, and that they have a smaller skull and finer skeleton than the pair-fed controls.

Histological studies [6] revealed striking differences between the bones of normal and low calcium rats. The contrast was especially well shown in the cortical bone of the tibial diaphysis where, in the depleted animals, porosis was very pronounced. The compact lamellation present in the normal bone was absent in the bones from the calcium-deficient animals, except for very limited thin periosteal and endosteal shells. Between these narrow periosteal and endosteal zones of almost normal appearing bone there were trabeculae of apparently poorly calcified osseous tissue. The nuclei of the osteocytes found in this tissue are much larger and stain more lightly with hematoxylin than those of the typical osteocyte. These cells were embedded in a fibrous matrix arranged in disorderly fashion, suggesting that the normal Haversian systems had been completely disrupted. Between the trabeculae a very cellular soft tissue had replaced the bone matrix. These areas showed blood vessels engorged with blood, and small hemorrhagic zones appeared occasionally. Many osteoclasts were observed with their cytoplasmic processes continuous with the remaining bone-trabeculae, resembling somewhat the condition produced by certain stages of dosage with parathyroid hormone. Also present in the resorption areas were many cells indistinguishable from fibroblasts as well as many typical osteoblasts, and where communications had been established with the marrow cavity, myeloid tissue had penetrated between the trabeculae.

Exophthalmos has been observed in rats that have been on the diet for as long as 14 weeks. This is probably a symptom of their higher metabolic rate. In the later stages of the deficiency, the animals may suffer a ringed-eye dermatitis, and several rats were observed to have developed a cataract of one eye.

Histological examination of animals suffering paralysis shows hemorrhagic areas in the following regions of the central nervous system: cerebrum, medulla, brain stem, about the choroid plexus of the third ventricle, in the lower right temporal pole of the brain, and, only occasionally, in the spinal

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[a] We are indebted to Dr. William R. Lyons for the description of the histologic picture of the bones.

cord. Evidence of hemorrhage is found in the lung, liver and kidney. There are small interstitial blood leaks in the voluntary muscle. The other organs examined were found to be normal.

In the brain no abnormality has been observed in the cerebellum. Special myelin staining shows no evidence of demyelinization of any part of the nervous system. However, the spinal cord itself is very soft and gelatinous in consistency on gross comparison with the normal. Practically no cellular reaction is found in the area of the intracerebral hemorrhages, whereas the medulla and choroid plexus of the third ventricle show hemosiderin phagocytosis.

Examination of the intestines reveals the presence of many brass-colored (hematoxylin-eosin stain) particulate bodies, located in the villous capillaries, in the intervillar spaces, and at the muco-submucosal junction of the large and small intestines. The particles are only occasionally phagocytized in these regions of the intestinal wall, but a diffuse deposition of the bodies is also observed in the fixed sub-mucosal macrophages. The particulate matter does not give the reaction for iron with the Prussian blue stain. There is much denudation of the intestinal wall of an occasional animal.

The black feces, encrusted nares, and traces about the uritoanal region of a bloody urine found in animals suffering from prostration and paralysis are, therefore, external evidence of hemorrhages that have occurred within the body.[7]

In explanation of the hemorrhagic state, it may be pointed out that calcium ion has long been associated with cell permeability, its effect on capillaries being to increase vascular tone and to decrease permeability by causing capillary constriction (Cantarow, '33). Eppright and Smith ('37) attributed the observed blood extravasation from the nares on a salt-free diet to a capillary fragility due to the deficiency of calcium. Histological examination indicates that possible alterations in the

[7] Because of the well-known fact that vitamin C deficiency causes hemorrhages in susceptible animals, experiments were carried out to determine the effect of the addition of ascorbic acid on the hemorrhagic condition suffered in calcium deficiency. No alleviation of the condition produced by a lack of calcium was found on feeding the experimental rats an abundant amount of ascorbic acid.

blood vessel walls are produced by our calcium-deficient regime.

8. Urine

A striking characteristic of the severe calcium deficiency is the output of a very large volume of urine. In one trial the deficient animals averaged an excretion of about 25 cc. of urine per day as against 8 cc. for the controls. The average specific gravity was 1.02 and 1.06, respectively. Microscopic examination showed the presence of some erythrocytes and very many

TABLE 2

Comparison of urinary volume and specific gravity in normal and calcium-deprived rats

		VOLUME	SPECIFIC GRAVITY
		cc.	
Calcium deficient	Rat 1	23.9 ± 1.9 [1]	1.02 ± 0.003
	Rat 2	24.5 ± 2.2	1.02 ± 0.002
Control	Rat 5	4.4 ± 0.6	1.08 ± 0.006
	Rat 6	10.5 ± 0.6	1.05 ± 0.006

[1] The figures represent the average of weekly values and their mean deviations of the mean for a period of 9 weeks on the experimental diet.

crystals in the urine of the calcium-deficient rats. A summary of the data on urinary volume and specific gravity is given in table 2. Data on the mineral composition of the urine will be published in connection with a study of the calcium balance in this condition. On the other hand, the output of feces by the calcium deficient rats is very scanty. The great reduction in the amount of calcium phosphate and calcium soaps in the intestinal tract is responsible for this.

CONCLUSIONS

The chief results of this investigation may be summed up in the following conclusions. A severe deficiency of calcium in the diet of the rat leads to musclar weakness and collapse rather than to tetany. The outstanding effect of the low calcium regimen is the susceptibility of the animal to hemor-

rhages. The bleeding may be the result of a disturbance of the role calcium plays in cell permeability, or may be due to changes in the vascular walls.

SUMMARY

1. Growing rats reared on a synthetic diet containing about 10 mg. calcium per 100 gm. food developed a condition characterized by the following syndrome: (a) retardation of growth; (b) decreased food consumption; (c) high basal metabolic rate; (d) reduced activity and sensitivity; (e) osteoporosis or low calcium rickets; (f) abnormal posture and gait; (g) susceptibility to internal hemorrhage which results in prostration, paralysis of the hind quarters (frequently fatal), encrusted nostrils, bleeding from the anus, and black, diarrheal feces; (h) a large increase in the volume of the urine; and (i) reduced span of life.

2. The hemorrhages and their consequences of prostration and paralysis are easily induced by a mild galvanic shock.

3. Rats suffering from the effects of calcium deprivation again become normal in appearance, reactions, and chemical composition a few weeks after they are supplied food with an adequate amount of calcium.

4. The injection of calcium salts almost invariably causes the death of the calcium-deprived animals within a few hours.

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SEVERE CALCIUM DEFICIENCY IN GROWING RATS[1]

II. CHANGES IN CHEMICAL COMPOSITION

MURIEL D. D. BOELTER[2] AND DAVID M. GREENBERG

Division of Biochemistry, University of California Medical School, Berkeley

FOUR FIGURES

A description of the symptoms and pathological changes produced by diets very low in calcium in growing rats has been given in the first paper of this series (Boelter and Greenberg, '41). The accompanying chemical changes in blood, tissues and bone form the subject matter of the present report.

With the exceptions noted below, the methods used for the analysis of blood, tissue and body constituents were the same as those employed by Tufts and Greenberg ('38).

RESULTS

1. Blood

There is a marked progressive lowering of the calcium concentration of the blood during the course of the extreme calcium deprivation. This is brought out in the curves of figure 1. The majority of the data were obtained from animals on a deficient diet containing approximately 10 mg. Ca per 100 gm. food. However, the three values, constituting curve 2, are from rats that received the 4 mg. Ca diet. These figures are among the lowest obtained and were reached much earlier in

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the course of the experiment. The ultimate result with either of the above calcium levels, however, is the same, so that ordinarily the less highly purified diet was employed.

The serum calcium starts to fall rapidly after the second week on the 10 mg. Ca diet, reaches its low level of around
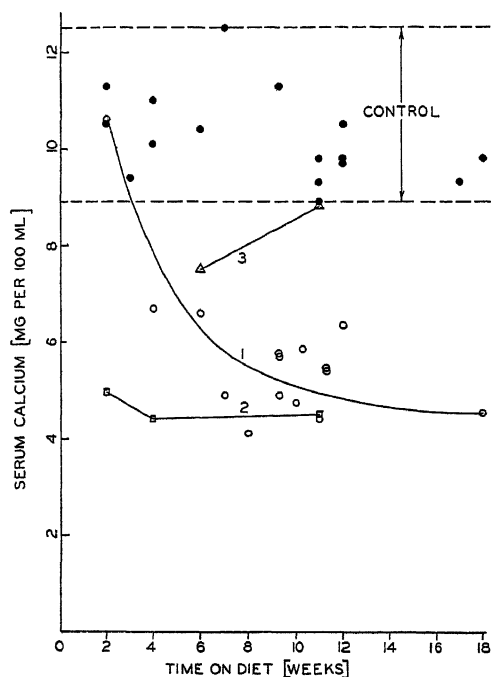

Fig. 1   The decrease in serum calcium concentration during calcium deprivation. Analysis made upon composite blood samples of two to four animals each. Results are expressed in milligrams of calcium per 100 cc. of serum. Curve 1 (open circles), about 10 mg. Ca per 100 gm. diet; curve 2 (open squares), about 4 mg. Ca per 100 gm. diet; curve 3 (open triangles), about 20 mg. Ca per 100 gm. diet; control range (solid circles), between the two horizontal dotted lines, about 550 mg. Ca per 100 gm. diet.

5 mg. per 100 cc. in about 8 weeks, and remains equally subnormal during the rest of the lifetime of the animal. The two points forming curve 3, in figure 1, represent analyses from rats receiving a ration which contained as much as 20 mg. Ca per 100 gm. food. They are decidedly higher than all the corresponding serum calcium values on the lower calcium-

containing diets. However, they are still below the lower limits of the control range. The time at which prostration and paralysis first can be produced by electrical stimulation corresponds with the attainment of a low serum calcium concentration.[3]

The low blood calcium levels are of interest because never has there been noted a case of tetany among the hundreds of animals reared by us that must have had a blood calcium of between 4 to 6 mg. per 100 cc., a concentration which is supposed to be the cause of "low calcium" tetany in parathyroidectomized animals.

Low values of the serum calcium concentration unaccompanied by any signs of tetanic convulsions have been reported in animals suffering from a variety of pathological conditions, including parathyroid deficiency (Godden and Duckworth, '35; Pal and Singh, '38; Salvesen and Linder, '23; Shohl and Bing, '28; Shohl and Wolbach, '36; Templin and Steenbock, '33; and Wade, '29). Many authors have come to the conclusion that factors other than calcium deficiency are involved in the production of tetany, and that a diminished level of serum calcium is not invariably associated with tetany.

The changes in inorganic serum phosphorus concentration are shown in figure 2. The inorganic phosphorus of the calcium-deficient animals falls at first more or less parallel to the calcium concentration, but it returns to the initial level after about 2 months on the calcium-low diet.

A summary of the blood levels of certain other constituents besides calcium and phosphorus is given in table 1. The serum and red corpuscle magnesium of the calcium-deprived rats remain within the normal range throughout the whole time of the experiment. The blood sugar and hemoglobin are the same for the deficient and normal rats. The serum phosphatase level, which might be expected to be altered because of

[3] Paralysis could be induced by electrical shock in parathyroidectomized rats maintained on the low calcium diet when their serum calcium concentration was normal.
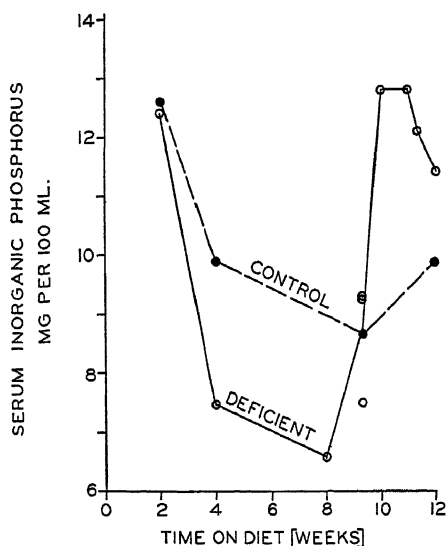
Fig. 2   Changes in serum phosphorus of animals reared on calcium deficient and normal diets. Data expressed as milligrams phosphorus per 100 cc. serum. Analysis upon pooled blood samples. Deficient (solid line and open circles), about 10 mg. Ca per 100 gm. diet; control (broken line and solid circles), about 550 mg. Ca per 100 gm. diet.

TABLE 1

Composition of the blood in calcium deficiency

CATEGORY OF INTEREST	DEFICIENT			CONTROL		
	No.	Range	Mean	No.	Range	Mean
Serum magnesium [1]	8	2.2–3.6	2.8	8	2.5–4.5	3.4
Red corpuscle magnesium [1]	8	4.3–9.9	7.6	8	6.3–8.8	7.8
Blood sugar [1]	2		84	2		85
Serum phosphatase [2]	3	108–123	115	5	98–132	112
Hemoglobin [3]	3	79– 80	80	4	79– 85	82

[1] Analytical values expressed in milligrams per 100 cc.

[2] Values expressed in tyrosine units (Greenberg, Lucia and Weitzman, '40). Several determinations by Bodansky's method ('33) yielded average values of 24.9 for deficient and 21.5 for control rats.

[3] Values expressed in per cent according to the Newcomer method ('23), (100% = 16.9 gm. hemoglobin).

the defects in the skeleton, was found also to be essentially the same as that of the calcium-supplied controls.

In these analyses blood sugar was determined by the method of Miller and Van Slyke ('36), phosphatase by the method of Greenberg, Lucia and Weitzman ('40) with disodium phenyl phosphate as substrate, and, in several instances, by Bodansky's method ('33) with sodium-β-glycero-phosphate as the

TABLE 2

Calcium, magnesium, and phosphorus content of certain tissues in extreme calcium deficiency. Data in milligrams per 100 gm. fresh tissue

	DEFICIENT				CONTROL			
	No.	Range	Mean	σ^1	No.	Range	Mean	σ^1
Calcium								
Muscle	19	3.1–19.2	7.2	0.9	17	4.0–16.8	7.9	1.0
Kidney	24	1.0–10.9	5.6	0.5	24	3.7–10.0	6.2	0.3
Heart	20	3.0–28.0	10.9	1.3	19	2.7–19.7	8.1	0.9
Magnesium								
Muscle	15	20.7–36.0	30.4	1.1	14	19.8–38.6	30.2	1.4
Kidney	18	18.9–27.1	23.6	0.5	18	15.1–37.9	23.8	1.2
Heart	14	19.8–36.9	25.8	1.2	13	17.6–25.9	22.3	0.75
Phosphorus								
Muscle	14	86–142	110	3.5	4	81–130	108	10
Kidney	14	117–279	153	10	7	123–213	153	11
Heart	13	86–142	110	5	3	87–134	113	—
Liver	17	100–164	140	4	7	88–148	120	7
Brain	15	124–170	143	4	6	123–174	149	7

[1] σ = mean deviation of the mean = $\sqrt{\dfrac{\Sigma(\overline{X}-X)^2}{n(n-1)}}$

substrate. Hemoglobin levels were determined by the Newcomer method ('23).

The only striking alteration, therefore, that has been observed in any of the blood constituents, is the reduction of the serum calcium concentration.

2. Soft tissues

Table 2 contains a statistical summary of the calcium, magnesium, and phosphorus contents, respectively, of certain of the soft tissues. These represent analyses at various stages

of the deficiency covering the time period from about 14 to 84 days. No striking changes from the normal are noted in the amount of calcium in the body tissues at any time during the progress of the deprivation. The results indicate that there may be some trend toward depletion of the calcium content of the muscle and kidney, but the variability is such that the
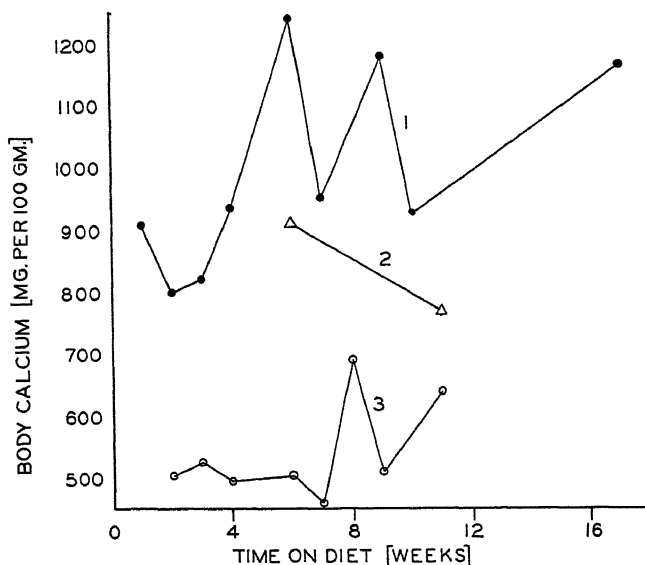

Fig. 3 Variation in calcium content of whole rat carcass with time during calcium depletion. Data are expressed in milligrams calcium per 100 gm. fresh carcass. The points represent weekly averages of analytical data. Curve 1 (solid circles), control, about 550 mg. Ca per 100 gm. diet; curve 2 (open triangles), about 20 mg. Ca per 100 gm. diet; curve 3 (open circles), about 10 mg. Ca per 100 gm. diet.

difference between the calcium deficient and control animals is not statistically significant. On the other hand, the calcium is tenaciously retained by the heart.

3. Mineral content of the whole body

The changes in body calcium are shown in the curves of figure 3. The points of the curves represent the average calcium percentage of groups of animals sacrificed at weekly intervals. After as little as 2 weeks on the calcium-low diet,

the body calcium is already reduced to about two-thirds the content of the control rats. Between 8 and 12 weeks on the diet, there is a noticeable increase in the per cent calcium of the deprived-rat carcass. The body calcium content of the control rats increases with growth, so that at 6 to 7 weeks the deficient level becomes actually less than half of the normal.

On the diet of 4 mg. Ca per 100 gm. food, the calcium depletion is even more drastic, the body calcium reaching the extremely low value of 313 mg. per 100 gm. of carcass in the rat reared for 11 weeks on this diet. This is considerably below the values found on the usual deficient diet at any time. On the 20 mg. Ca diet, depletion of the body calcium is very much slower, so that at 6 weeks on the diet, the body calcium is nearly within the control range.

The average values of the per cent magnesium in the bodies of the calcium-deficient animals are always below those of the normal rats of the same age. The lowered total body magnesium reflects the low content of magnesium in the bones of the calcium-depleted animals. The changes in body magnesium are shown in figure 4. During the initial stages of calcium depletion, there is an increase in whole body magnesium, but after 2 months on the calcium-low regime, the per cent of magnesium decreases, paralleling the drop observed in the control animals.

4. Composition of bone

A statistical summary of the chemical data obtained from the analysis of the bones of the deficient and control animals is recorded in table 3.

The bones of the calcium-deprived animals are poorly calcified, but the composition of the bone mineral is not essentially altered. The poor degree of mineralization of the bones of the deficients is indicated by the ash content being about 40% below that of the controls. Because of the low ash content, the percentage of calcium in the deficient bones is only about one-half that of the controls. The magnesium and phosphorus are about one-third lower than the normal. In percentage of bone
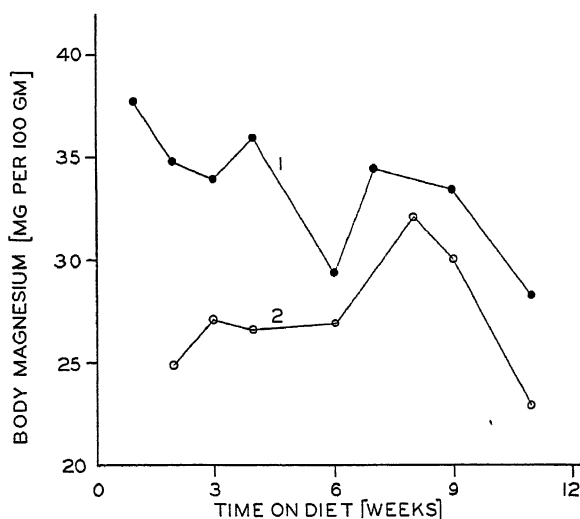
Fig. 4   Changes in total body magnesium during the course of calcium depletion. Data are expressed as milligrams magnesium per 100 gm. fresh carcass and are obtained from the weekly averages of the analytical values. Curve 1 (solid circles), control, about 550 mg. Ca per 100 gm. diet; curve 2 (open circles), about 10 mg. Ca per 100 gm. diet.

TABLE 3

Composition of bone

| | ASH | CALCIUM | | MAGNESIUM | | PHOSPHORUS | |
		Ash	Bone	Ash	Bone	Ash	Bone
	%	%	%	%	%	%	%
			Calcium deficient (21 analyses, 14–84 days on diet)				
Range	21.6–53.9	18.2–40.6	6.7–19.9	0.39–1.96	0.15–0.62	10.1–20.5	3.6–9.8
Mean	39.9	29.7	11.8	0.94	0.35	16.9	6.9
σ^1	1.7	1.2	0.7	0.09	0.03	0.5	0.4
			Control (11 analyses, 7–84 days on diet)				
Range	54.0–69.2	32.3–43.7	18.6–28.3	0.56–0.90	0.37–0.59	13.4–20.3	9.0–13.1
Mean	65.0	38.1	24.7	0.75	0.48	16.3	10.5
σ^1	1.3	1.3	0.9	0.04	0.03	0.6	0.4
			Recovered [2]				
Range	72.7–73.8	29.6–32.4	21.5–23.9	0.46–0.48	0.33–0.37	12.3–12.9	8.9–9.5
Mean	73.2	31.0	22.7	0.47	0.35	12.6	9.2

[1] σ = mean deviation of the mean = $\sqrt{\frac{\Sigma(\overline{X}-X)^2}{n(n-1)}}$

[2] Data for two rats that were reared on the control diet for about 22 weeks after having been deprived of calcium for 10 weeks following weaning.

ash, the mean calcium value is only 16% below normal, the magnesium about 25% higher than the control, and the phosphorus is unchanged.

Analysis of the bones of two animals given the control food for 22 weeks after they had been 10 weeks on the calcium-low diet following weaning revealed a normal percentage of ash and calcium, a significant reduction in phosphorus, and a very low content of magnesium.

SUMMARY

1. Severe calcium deprivation causes a rapid reduction in the serum calcium concentration, and a decrease in the calcium and magnesium content of the whole rat carcass. The serum inorganic phosphorus, magnesium, and phosphatase, red corpuscle magnesium, and the blood sugar do not deviate from normal. There is the usual content of hemoglobin in the blood. The soft tissues maintain their normal mineral content.

2. The bones of the deficient animals are greatly demineralized. The per cent of ash and calcium of the bone is only about one-half the normal. There is a lesser decrease in the phosphorus and magnesium percentage. In the bone ash, the contents of calcium, magnesium, and phosphorus are slightly low, high, and normal, respectively.

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STUDIES ON RIBOFLAVIN AND THIAMIN IN THE RUMEN CONTENT OF CATTLE [1]

CHAS. H. HUNT, C. H. KICK,[2] E. WISE BURROUGHS, R. M. BETHKE,
A. F. SCHALK AND PAUL GERLAUGH

Department of Animal Industry, Ohio Agricultural Experiment Station, Wooster

Bechdel, Honeywell, Dutcher and Knutsen ('28) in connection with studies on the synthesis of the vitamin B complex in the rumen of the cow reported that the fermented rumen content contained more of the vitamin B complex than the feed of the animal. These investigators also found that one bacterium of the genus Flavobacterium was about 90% predominant in the rumen microflora and that the feeding of the dead bacteria to rats on a vitamin B complex free ration resulted in increased growth. From these findings the authors concluded that the vitamin B complex was produced in the rumen of the cow by bacterial action and that the results offered an explanation as to why Bechdel, Eckles and Palmer ('26) were able to grow cattle to maturity and have them produce normal offspring on a ration that carried an insufficient amount of the vitamin B complex to support growth and well-being in rats. Since this work was done, it has been shown that the vitamin B complex, as originally postulated, is made up of several factors, parts of which have been chemically identified.

The fact that Bechdel and associates ('28) found a chromogenic bacterium to predominate in the microflora of the rumen suggested to us that possibly riboflavin was being synthesized in the rumen by bacterial action and not vitamin B_1 (thiamin) alone.

In connection with another study that is in progress at this institution, which makes use of an artificial rumen fistula in steers, the opportunity was afforded to study the possible synthesis of some of the fractions of the vitamin B complex in the rumen of the animal. The present report deals with riboflavin and thiamin of the feed and rumen content.

EXPERIMENTAL

Biological assays

The experimental procedure consisted of feeding weighed amounts of the feed the steers received or the dried content of the rumen to rats, as sources of riboflavin and thiamin. The rats were confined to wire cages when 35 to 45 gm. in weight and fed a vitamin B complex free ration, consisting in percentage composition of vitamin-free casein [3] 20; sucrose, 71; salt mixture, 4; hydrogenated cottonseed oil,[4] 3; and cod liver oil, 2.

For the riboflavin studies, the above ration was supplemented with daily additions of 10 µg. of thiamin and a water soluble fraction of yeast [5] from which the greater part of the riboflavin had been removed. The amount of yeast extract fed daily was equivalent to 1.5 gm. dried yeast. This amount of yeast extract plus thiamin resulted in an average gain of 10 gm. in forty-six rats during the assay period of 6 weeks. When adequate amounts of riboflavin (40 µg.) were fed in addition to the yeast extract and thiamin, average gains of about 20 gm. per week for 6 weeks resulted. The yeast extract in amounts of 0.25 gm. dried yeast daily also cured acrodermatitis in rats.

When the rats had attained a satisfactory weight (plateau in weight curve in 2 to 3 weeks) as evidenced by daily weighings, they were transferred (equal number of males and females) to individual cages and the finely ground samples of feed or dried rumen content were fed in separate dishes as the source of riboflavin. A negative control group was included

[3] Labco.
[4] Crisco.
[5] Northwestern Yeast Co., Chicago, Ill.

in each trial. At the same time different levels of synthetic riboflavin were fed in addition to the yeast extract and thiamin to obtain a calibration or reference curve (table 1) which was used to calculate the riboflavin content of the products under study (table 2). To assure that the growth response induced by feeding the dried ingesta in these studies was a measure of its riboflavin content and not of other growth factors, a group of rats was fed 600 mg. of dried (16 hours) ingesta daily from trial 5 in addition to the yeast extract, thiamin and 40 µg. riboflavin. The results presented in table 1 show no growth increase from the feeding of the dried ingesta.

TABLE 1

Weight increments in rats fed varying amounts of riboflavin

SUPPLEMENT	NUMBER OF ANIMALS	AMOUNT FED DAILY	AVERAGE INCREASE IN WEIGHT 6 WEEKS	AVERAGE INCREASE IN WEIGHT OVER NEGATIVE CONTROLS
		$\mu g.$	$gm.$	$gm.$
	8	2.5	32	25
Positive control	8	5.0	53	46
Synthetic ribo-	8	10.0	83	76
flavin (Merck)	8	20.0	111	104
	8	40.0	126	119
600 mg. ingesta	5	40.0		117
Negative controls	8	0.0	7	

In the thiamin studies the above vitamin B complex free ration was supplemented daily with 600 mg. of autoclaved yeast as the source of the vitamin B complex factors other than thiamin. In trial 5 the autoclaved yeast was replaced with 1.5 cc. of the yeast extract (1.5 gm. yeast) and 40 µg. of riboflavin. Rats which received either the autoclaved yeast or yeast extract plus riboflavin all died of polyneuritis in the course of 3 to 4 weeks. When the growth curves of the rats had exhibited a weight plateau (3 to 4 weeks) the animals were transferred (equal number of males and females) to individual cages and given weighed amounts of the finely ground feed or dried ingesta as the source of thiamin. The supplemental feed-

ing was continued for 6 weeks. Negative control groups were included in each series.

The steers used in trials 1, 2, 3, 4, 6, and 7 were mature animals and had been on their respective rations for several weeks before the samples of ingesta were collected. The daily ration in case of trials 1, 2, 3, and 4 consisted of 8.0 pounds of yellow corn, 8.0 pounds alfalfa hay, and 1.5 pounds of protein supplement. In trials 6 and 7 the steers were fed alfalfa hay exclusively. Two young steers weighing about 400 pounds were used in trial 5 and each was fed daily 2.5 pounds yellow corn, 3.5 pounds chopped alfalfa hay, and 0.5 pound protein supplement. One of these steers was fed its portion of corn whole and the other ground. Samples of ingesta were collected from each animal after they had been on the above ration for 2 weeks, then the rations were reversed and another sample of ingesta taken from each animal after 10 days of feeding. The ingesta from the two animals fed whole corn and the same animals fed ground corn were then combined for drying and feeding.

Method of collecting samples

In all trials except trial 5 the content of the rumen was sampled by removing 15 to 20 pounds of the ingesta from various parts of the rumen at different intervals after feeding. The sampling in all but three trials extended over 3 or more days. After sampling and compositing, the ingesta were dried in shallow pans before an electric fan in a dark room. In some cases part of the composited ingesta were incubated for 12 or 48 hours at 37°C. and then dried. The samples in trial 5 were obtained by removing the entire rumen content of the steer at each collection period. The ingesta were thoroughly mixed, sampled, and the remainder returned to the rumen. The fresh samples of ingesta were treated with 95% ethanol so that the final concentration was about 45% by weight and then stored in a cool, dark place until all samples had been collected and composited, after which they were dried by the same method as in the other trials.

The feed in all trials was sampled on the day before and during the days when collections of ingesta were made. The samples for each trial were then finely ground and composited in the same proportion as they were fed to the animal. All samples of feed and dried rumen content were stored in the dark.

RESULTS AND DISCUSSION

The results of the riboflavin studies are presented in table 2. In all instances (trials 1, 3, 4, and 5) where the steers had been fed a ration of yellow corn, alfalfa hay, and a protein supplement, the dried ingesta which had been in the rumen 12 or 16 hours showed a greater riboflavin content than the feed. In two out of the three trials (trials 2, 4, and 5), when the corn-hay-protein supplement ration was fed, the 4-hour dried ingesta sample showed an increased riboflavin content when compared with the feed. The incubation of the 4-hour ingesta sample in two trials showed an increase in riboflavin content when incubated for 48 hours (trial 2) but no increase when incubated for 12 hours as in trial 4. The 16-hour ingesta sample (trial 3) incubated for 48 hours did not show an increase in riboflavin due to incubation.

It is of interest that in both trials (6 and 7) where alfalfa hay served as the only feed of the steers no increase in the riboflavin content of the dried rumen content was observed either in the 4- or 16-hour sample. Furthermore, there was no increase in the riboflavin content of the 4-hour ingesta (trial 7) sample after incubating for 48 hours at 37°C. Kick and associates ('38) have reported that the rumen content of steers fed alfalfa hay exclusively is alkaline in reaction, whereas if grain and a protein supplement are fed with alfalfa hay the rumen content is acid in reaction. Whether the reaction of the rumen content or some other factor was concerned in the results obtained on the two different types of rations used in these studies is not clear and requires further investigation. The results, in general, show that a synthesis of riboflavin takes place in the rumen of the steer when a mixed ration of corn, alfalfa hay, and a protein supplement is fed. These observa-

TABLE 2

The comparative riboflavin content of the feed and the dried ingesta of the rumen

TRIAL	MATERIAL	TIME[1] IN RUMEN	TIME INGESTA INCU-BATED AT 37°C.	DAYS INGESTA COL-LECTED	RATS	AMOUNT FED DAILY	AVERAGE GAIN OVER CON-TROLS	ESTIMATED RIBO-FLAVIN CONTENT PER GRAM
		hours	*hours*	*no.*	*no.*	*mg.*	*gm.*	*μg.*
1	Feed				6	600	29	5
	Ingesta	12		1	6	600	42	7
2	Feed				8	300	20	7
	Ingesta	4		1	8	300	20	7
		4	48	1	8	300	34	11
3	Feed				8	300	15	5
		16		3	8	300	27	8
	Ingesta	16	48	3	8	300	25	8
		16		9	8	300	25	8
4 [2]	Feed				8	300	15	5–6
					8	600	37	
	Ingesta	4		3	8	300	28	9
		4	12	3	8	600	52	
		4		3	8	600	52	9
	Ingesta	16		3	8	300	47	16
		16		3	8	600	75	
5	Feed				8	300	14	4–5
					8	600	27	
	Ingesta[3]	4		3	8	300	21	7–9
		4		3	8	600	54	
		16		3	8	300	31	10–13
		16		3	8	600	71	
	Ingesta[2]	4		3	8	300	30	8–9
		4		3	8	600	50	
		16		3	8	300	33	10–12
		16		3	8	600	61	
6 [4]	Feed				8	300	31	10–12
					8	600	65	
	Ingesta	4		3	8	300	17	5–6
		16		3	8	300	8	4–6
		16		3	8	600	37	
7 [4]	Feed				8	500	38	7–8
	Ingesta	4	48	1	8	500	35	7–8
		4	48	1	8	500	32	6–7

[1] Interval after last feeding when sample was withdrawn from rumen.

[2] Ground corn.

[3] Whole corn.

[4] Alfalfa hay ration.

tions are in accord with the findings of McElroy and Goss ('39) and offer a possible explanation as to why Johnson, Loosli and Maynard ('40) did not obtain improved growth in calves fed yeast or liver as supplements to a purified ration devoid of riboflavin.

The results of the thiamin studies presented in table 3 show that the dried ingesta of the rumen taken 12 or more hours after feeding contain less thiamin than the feed given the

TABLE 3

The comparative thiamin content of the feed and the dried ingesta of the rumen

TRIAL	MATERIAL	TIME[1] IN RUMEN	TIME INGESTA INCUBATED AT 37°C.	DAYS INGESTA COLLECTED	RATS	AMOUNT FED DAILY	AVERAGE GAIN OR LOSS IN WEIGHT	RATS WITH POLYNEURITIS
		hours	*hours*	*no.*	*no.*	*mg.*	*gm.*	*%*
1	Feed				6	400	20	
	Ingesta	12		1	6	400	−2	66
2	Feed				8	400	19	
	Ingesta	4		1	8	400	28	
		4	48	1	8	400	15	
3	Feed				8	400	21	
	Ingesta	16		3	8	400	10	30
		16	48	3	8	400	1	50
		16		9	8	400	2	
4	Feed				8	400	19	
					8	800	41	
	Ingesta	4		3	8	400	12	37
		4		3	8	800	30	
		4	12	3	8	800	34	
	Ingesta	16		3	8	400	9	25
		16		3	8	800	38	
5	Feed				8	400	21	
					8	800	72	
	Ingesta[2]	16		3	8	400	−10	37
		16		3	8	800	5	50
	Ingesta[3]	16		3	8	400		100
		16		3	8	800		100
7[4]	Feed				6	500	16	
	Ingesta	4		3	6	500	27	
		4	48	3	6	500	7	

[1] Interval after last feeding when sample was withdrawn from rumen.
[2] Whole corn.   [3] Ground corn.   [4] Alfalfa hay ration.

steer. The 4-hour ingesta samples, on the contrary, showed an increased thiamin content in two of the three trials. This suggests either that the thiamin was rapidly absorbed by the animal or that it was in part destroyed, since the incubation of the fresh ingesta in four trials for 12 or 48 hours resulted in a decreased thiamin content. Further studies are required to determine which of these possibilities is correct.

CONCLUSIONS

The results of these studies show that riboflavin is synthesized in the rumen of the steer when the animal is fed a ration of yellow corn, alfalfa hay, and a protein supplement.

Evidence was also obtained that the dried ingesta of the rumen of the steer fed exclusively on alfalfa hay contained less riboflavin than did the hay.

The data also indicate a greater thiamin content of the dried ingesta of the rumen removed 4 hours after feeding than in the feed, and a decreased content 12 or 16 hours after feeding.

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A STUDY OF THE NEED FOR COBALT IN DOGS ON MILK DIETS [1]

DOUGLAS V. FROST, C. A. ELVEHJEM AND E. B. HART

Department of Biochemistry, College of Agriculture, University of Wisconsin
Madison

THREE FIGURES

Cobalt was found to be essential in the nutrition of sheep by Underwood and Filmer ('35), Lines ('35) and of cattle by Neal and Ahmann ('37) and Filmer and Underwood ('37). In all of the above cases anemia was found to occur in the animals on natural rations grown in areas known to have a very low content of cobalt in the soil. Underwood and Elvehjem ('38) attempted to demonstrate a cobalt deficiency effect in rats on milk diets and concluded that, if a cobalt requirement exists at all, it must be less than 6 µg. per kilogram of body weight per day.

Since some of our growing dogs failed to attain normal blood values over several months' time with iron and copper therapy, we became interested in the possibility that cobalt might function at that point to stimulate hematopoiesis.

EXPERIMENTAL

Attempts to produce cobalt deficiency. Nutritional anemia was produced in a litter of six Beagle puppies by the method of Potter et al. ('38) and Frost and associates ('40). In this case the pups were obtained at 2 weeks of age and were placed with the mother on raised screens. Only cows' milk was fed

to the mother for the next 2 weeks, at the end of which time the pups were weaned to milk. The development of anemia in these dogs was very rapid. The type of therapy varied with the different dogs, but two typical results are shown in figure 1. One of the dogs was given 20 mg. of iron and 2 mg. copper daily when the hemoglobin level reached 6 gm. per 100 cc. of blood. An immediate response occurred, but when the hemoglobin reached about 11 gm., there was little if any additional improvement. The level was maintained for about 10 weeks. When 4 mg. of cobalt per day were added in addition to the
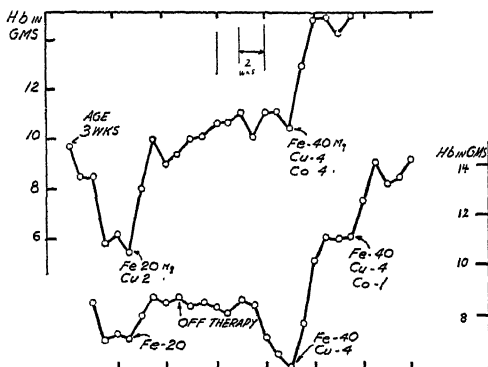
Fig. 1   The additive effect of iron, copper and cobalt in blood building in young dogs.

iron and copper, there was an immediate increase in hemoglobin and normal levels were reached. The other dog was given a preliminary treatment with iron alone to deplete the copper reserve. When iron and copper were given at levels of 40 mg. and 4 mg. respectively, the typical response was obtained but a plateau was again observed at 11 to 12 gm. hemoglobin. The dog showed a rapid increase in hemoglobin when 0.1 mg. of cobalt was given per day. The calculated hemoglobin production during the first 2 weeks of cobalt supplementation was many times that produced during any 2 weeks immediately preceding the cobalt feeding.

In order to check the apparent stimulating effect of cobalt for young dogs we repeated the experiment with another litter

of eight mongrel Spaniel pups. This litter was also obtained at 2 weeks of age with the mother and was weaned to cows' milk at 4 weeks of age. When the pups were 7 weeks old, they were quite uniformly anemic. The hemoglobin level in each case was between 4 and 5 gm. per 100 cc. of blood. Four pups were then selected for a comparison of the rate of hemoglobin building with and without cobalt. Each of one pair of dogs was fed 10 mg. of iron plus 2 mg. of copper daily and each of another pair was fed the same combination plus 0.5 mg. of cobalt daily. During therapy 6 cc. samples of blood were taken from the jugular vein every fourteenth day.
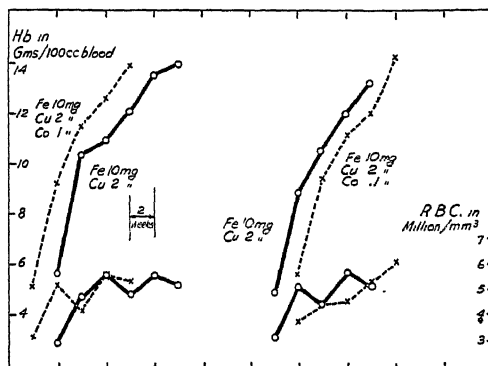
Fig. 2 Comparison of rate of blood building in young dogs fed iron and copper and iron, copper and cobalt from the beginning of therapy.

Figure 2 shows the uniformity of the blood picture as regards hemoglobin and red blood cell count in all cases. Hematocrit readings ranged from 16 to 19% at the start of therapy and 38 to 41% at the end of the 16 weeks period of therapy. As an added precaution against cobalt contamination in this experiment the ferric chloride was extracted with ether as follows: Analytical reagent iron wire (Mallinckrodt) was dissolved in C.P. concentrated HCl and extracted continuously in a Kutscher-Steudel apparatus with ether. Superoxol was used to oxidize the iron completely, but a small amount of ferrous chloride was added back to prevent the formation

of explosive ether peroxides during the extraction. The ether extract containing cobalt-free ferric chloride was freed from ether and made to the proper dilution by analysis.

Effect of hemorrhage. In order to determine whether or not a cobalt deficiency could be produced by repeated production of anemia through hemorrhage, or by long-continued nutritional anemia, the following experiments were performed. Two litter mate pups were made anemic by the usual procedure. One of them was given 10 mg. of iron, 2 mg. of copper, and 1 mg. of manganese daily for 13 weeks. At the end of this time the hemoglobin of the blood had reached a normal level. Therapy was discontinued and the dog was subjected to severe bleeding by phlebotomy. When anemia had again been produced, iron, copper and manganese were once more introduced. Again the anemia was rapidly and completely cured. A third anemia was produced by bleeding and was once more completely cured by iron, copper and manganese therapy.

The litter mate to the above dog was fed iron alone at weaning but was restricted to a total intake of 420 mg. of iron. The dog was then fed milk alone for several months and finally bled to produce a severe copper deficiency. At this point no response was shown to iron feeding. After 3 weeks copper was added to the iron therapy. The response to copper was not complete and the hemoglobin level remained at 11 gm. per 100 cc. of blood. The interesting observation was made at this time that the red blood cell count fell from 8 million per cubic millimeter of blood to 5.4 million during the period of copper therapy. When cobalt was added to the iron, copper and manganese at a level of 0.5 mg. per day, a sharp rise in hemoglobin and red blood cell count ensued. The hemoglobin level rose from 11 to 15.4 gm./100 cc. and the cell count from 5.4 to 9.4 M/c.mm. in a period of 3 weeks (fig. 3). Another dog made severely anemic by phlebotomy and depleted of copper showed an actual decrease in all blood values during iron and copper therapy, but exhibited a rapid increase when cobalt was added at a level of 0.1 mg. per day.

Addition of cobalt to dogs severely deficient in copper was without benefit even when adequate iron was given. In general the effect of cobalt seemed to be supplementary to that of iron and copper in those instances where copper and iron alone did not suffice to stimulate normal blood building.

Determinations of reticulocyte count, hematocrit per cent and hemoglobin were made at regular intervals throughout these studies to determine whether dogs on mineralized milk diets show normal blood cytology. The values obtained compare so closely with those described by Scarborough ('30), Mayerson ('30), and Morris et al. ('40) for large numbers of
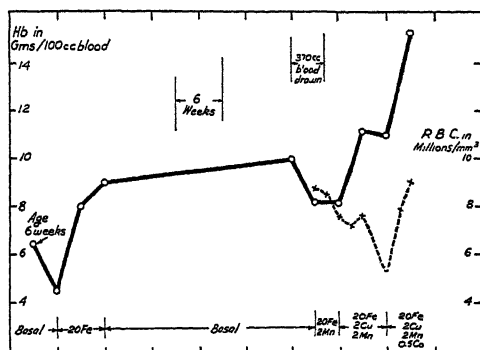
Fig. 3 Effect of cobalt addition to iron and copper therapy after long-continued nutritional anemia and hemorrhage.

dogs on regular kennel diets that it is not necessary to report them here. Let it suffice to say that the maintenance of hemoglobin at subnormal level in certain of our younger dogs is not to be confused with the normal physiological lag in arriving at adult hemoglobin levels shown by most species of animals in the rapid growing stage, and described for dogs by Morris et al. ('40). Comparison of our figures with those of Morris indicated that many of our dogs attained normal adult blood levels at a comparatively early age. Responses to cobalt appeared to be independent, so far as we could tell, of the age of the dogs.

DISCUSSION

The results of the above experiments indicate that a cobalt deficiency anemia is extremely difficult to produce uniformly in dogs on milk diets. The stimulating effect of cobalt which we have observed in certain dogs appeared quite real and unequivocal. Possibly this effect is non-specific and would be produced by other elements as well; however, the well-known capacity of cobalt to stimulate a polycythemic response in many species suggests that here we are dealing with a specific function of cobalt.

The cobalt content of milk is very low. Wright and Papish ('29) and Blumberg and Rask ('33) claim that milk is "spectrographically free" of cobalt. Underwood and Elvehjem ('38) used the method of Kidson, Askew and Dixon ('36) and obtained a mean of 11 μg. of cobalt per liter of milk in five analyses controlled by blanks and recovery tests. Results of several analyses which we made on 500 cc. samples of milk checked the results of Underwood and Elvehjem fairly well when the color comparisons were made visually. When the photometric estimation method of MacPherson and Stewart ('38) was used with the Evelyn photoelectric colorimeter, satisfactory recoveries of cobalt were not obtained on milk. Calcium is known to interfere with the accurate colorimetric determination of cobalt and this may explain the paucity of reports in the literature regarding the cobalt content of milk. For purposes of interpretation of our experiments we can safely say that the cobalt content of milk is low. Nothing is known of the possible variation in cobalt content of milks from different sources.

If we consider the cobalt content of milk to be from 10 to 20 μg. per liter, the cobalt intake of dogs on milk diet would be about 1.5 to 3 μg. per kilogram of body weight per day. Thus a 25 kg. dog might ingest as much as 0.075 mg. of cobalt daily from the milk alone. Since the daily requirement of a 25 kg. sheep has been placed at only 0.1 mg. per day, it appears that we may be operating at a level close to the minimal requirement. Only the use of purified diets or of foods from

cobalt deficient areas will permit a decision as to whether cobalt is generally essential for normal function of hematopoietic organs.

Use of cobalt in treatment of human anemia in infants by Kato ('37), in children by Waltner ('30) and in adults by Baxter ('39) and Cronin ('39) appeared successful only in the hands of the first author. In most of these cases rather large amounts of cobalt were used and in some of them the size of the dose would border on the predicted polycythemia-producing level for humans. A level of 0.15 mg. of cobalt per day is about the detectable polycythemia-producing level in rats. A polycythemic response in dogs has been obtained with a dose of 2 mg. per kilogram of body weight per day. The general biologic property of cobalt to produce polycythemia in animals may be quite unrelated to the essential role it plays in the nutrition of certain species. In any case the part played by cobalt in hematopoiesis is unique and continued study of its effects should throw some light on the intricate mechanisms involved in blood formation.

SUMMARY

In many cases iron and copper suffice with milk for normal hemoglobin building in dogs. The addition of small amounts of cobalt to iron and copper therapy stimulated hematopoiesis in certain dogs in which the rate of blood formation appeared unusually slow. About one-half of the dogs studied showed a cobalt deficiency as evidenced by hematopoietic responses to cobalt at a minimum level of 0.1 mg. per day.

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IRON BALANCES ON FOUR NORMAL PRE-SCHOOL CHILDREN [1]

THELMA PORTER

*Section of Home Economics, Department of Food and Nutrition,
Michigan Agricultural Experiment Station, East Lansing*

The thirty-five iron balances on normal pre-school children reported in this paper were a portion of the data collected in a study to determine the effect of a farina fortified with irradiated yeast on the mineral metabolism of the subjects. In the original plans for this study, interest centered around the effect of the vitamin-D-fortified farina on calcium and phosphorus utilization.[2] As the plans progressed, however, it was decided to take all the necessary precautions in managing the subjects and in collecting the specimens to allow for complete iron balance determinations. This was considered advisable because the period of observation was longer than any previously reported on this age group, as well as because possible interrelationships between iron and the vitamin D supplement might be observed.

EXPERIMENTAL PROCEDURE

The four children cooperating in this study were in fairly good nutritional condition and in good general health at the beginning of the study. There were three boys, O, R, and K in

[2] Calcium and phosphorus balances are now being prepared for publication.

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the group, and one girl, M. Subject O was 3 years old, 100 cm. tall and weighed 17 kg. R was also 3 years old, 100 cm. in height, and weighed 16 kg. Subjects K and M were 5 years old. K was 114 cm. tall and weighed 20 kg., while M was 112 cm. in height and 21 kg. in 'weight. At the beginning, mid-point, and end of the study, medical and physical examinations were given the children by Doctor Grissom, a member of The University of Chicago Nursery School staff. Hemoglobin percentages and red cell counts were recorded for each child.

The management of the subjects was carefully planned and supervised in every detail including an effort to prevent the intake of any form of iron from toys or other items in the environment so that intake of food iron would represent the total consumption of that mineral. No serious disciplinary problems or emotional disturbances arose to affect the progress of the study.

The customary diets of the subjects were reasonably adequate, as far as could be determined. They contained liberal quantities of milk and vegetables as well as some cod liver oil or viosterol as a supplement. During the experiment the diet was planned in a 7-day unit. An example of O's diet, with quantities of foods he ate, is given in table 1. This diet had a calculated average nutritive value of approximately 1300 calories, 45 gm. of protein, 1 gm. of calcium and of phosphorus, and 6 mg. of iron (Waller, '38). Using the average weight of O throughout the study, the calories averaged 76.3 per kilogram of body weight per day while the protein allowance was 2.63 gm. and represented about 13% of the total calories. Of the vitamins, the food furnished about 5000 I.U. of vitamin A, 75 mg. of ascorbic acid, 0.8 mg. of thiamin, and 1.5 mg. of riboflavin. Such quantities of these vitamins adequately meet the daily allowances suggested for children of this age by Stiebeling and Phipard ('39). The daily average vitamin D content of the unfortified basal diet was only about 40 I.U. In calculating this figure, minimum values for foods as given by Chaney and Ahlborn ('39) were used, because the

foods were winter foods. Each day the diet contained approximately 1 ounce (28 to 30 gm., dry weight) of either plain farina or farina fortified with a fraction of 1% of irradiated yeast. The inclusion of fortified farina resulted in amplifying the basal diet with approximately 120 I.U. of vitamin D per day. No additional vitamin or mineral supplements were given.

TABLE 1

Food list, in grams, for subject O for 7 days

EVERY DAY		DAY 1 ADDITIONS		DAY 2 ADDITIONS		DAY 3 ADDITIONS	
Milk	720	Egg	50	Bacon, cooked	10	Stew (beef)	75
Orange juice	100	Beef, ground	30	Beef, ground	60	Tomato-soup	100
Farina,		Tomatoes,		Spinach	40	Spaghetti	25
cooked	140	canned	60	Peas, creamed	75	Peaches, canned	90
Bread, whole		Potato, baked	40	Potato, baked	20	Prunes, stewed	100
wheat	20	Mousse, fruit	45	Pears, canned	90	Apple, E.P.	50
Crackers,		Apple sauce	100	Banana, E.P.	60		
graham	14			Apple, E.P.	50		
Butter	24						
Sugar	10						

DAY 4 ADDITIONS		DAY 5 ADDITIONS		DAY 6 ADDITIONS		DAY 7 ADDITIONS	
Bacon, cooked	10	Liver	30	Bacon, cooked	10	Stew (beef)	75
Spaghetti		Vegetable		Salmon,		Peanut butter	9
with beef	100	soup	150	canned	25	Bread, whole	
Cabbage	50	Crackers, soda	30	Potato, baked	20	wheat	10
Apricots	45	Beans, green	50	Beets	40	Beans, green	50
Jello	60	Potato, baked	20	Carrots	50	Peaches, canned	90
		Pears, canned	90	Pineapple,		Prunes, stewed	100
		Apple sauce	100	canned	60		
				Apple, E.P.	80		
				Banana, E.P.	30		

The diets of the other children contained these same kinds of food with minor adjustments in quantities to meet each child's needs. All children received milk, orange juice, and farina in the quantities listed in table 1. The food intake was kept as constant as possible for each subject and every necessary precaution for procuring a uniform food supply was exercised. All food combinations were made by weighed

recipes; and all foods were prepared in enamel or aluminum utensils with plated cutlery. Distilled water was used for cooking and drinking.

After a preliminary period of 3 days which permitted the establishment of a smooth running routine, the weighed diet and the collections were begun. The experimental calendar was made up of nine 7-day periods, a total of 63 days, beginning the latter part of December, 1932, and extending into February, 1933. Three subjects, O, R and M, served for all nine periods, while subject K served for eight of the periods. One 3-year-old and one 5-year-old, O and M respectively, began the experiment on diets containing 1 ounce per day of fortified farina. After remaining on this food for 21 days (three 7-day periods) they were given the basal diet with plain farina instead of the fortified product for 21 days, then returned to the original diet for 21 days. The other two subjects, R and K, reversed the type of diet in corresponding periods, that is, they began with 21 days on plain farina, followed with 21 days on fortified farina, and finished with 21 days on plain farina again.

For chemical analysis, one-tenth of the quantity of every food eaten was made into a "composite" for each subject and at the same time, all urine and feces for corresponding periods were saved. The food aliquots, as well as the feces acidified with iron-free hydrochloric acid and mixed with some alcohol, were dried to a constant weight in porcelain or Pyrex dishes at 80°C. Precautions were taken to keep these samples covered with parchment paper caps to protect them from dust and other sources of iron contamination during the drying. When dry, they were ground in porcelain mortars to homogeneous mixtures and stored in glass-topped bottles. The urine was preserved with the aid of 0.5% of iron-free hydrochloric acid and a thin film of toluene on the surface.

Aliquots of food, feces, and urine composites were dry ashed in duplicate in an electric muffle furnace at a temperature below dull red heat. The thiocyanate method as described by Stugart ('31) with some of the modifications sug-

gested by Leverton ('38) was employed for determining the iron in these ashes. All determinations were made colorimetrically on two aliquots from duplicate ash solutions, making a total of four readings on each unknown. In this report, results on the two ash solutions are used only when they agreed within approximately 5%.

In order to determine whether or not iron metabolism was changed by substituting for 1 ounce of ordinary farina an equal quantity of fortified farina containing 120 I.U. of vitamin D per day, certain tests for significant differences in average storage of iron on the two diets were applied. These included, first, the use of Fisher's extension of "Student's" t-test as developed by Brandt ('38) for biological experiments involving reversal or switchback trials with only one pertinent source of variability. In this case, a single change in character of basal diet was imposed on the subjects. This test was supported further by the use of an analysis of variance (Baten, '38).

DISCUSSION OF RESULTS

The medical records of the children during the period of study indicated gains in weight ranging from 6 ounces for K to 2 pounds and 3 ounces for M; and in height, from about $\frac{1}{2}$ inch for K to 1.7 inches for R during the 2-month period. With the exception of K's cold in period VII, when no data were collected for him, there were no illnesses or upsets which interfered with the experimental program. It will be noted that K made the poorest growth of any of the children during these 2 months.

Since this study was undertaken, the limitations of the balance technique as a means of studying iron utilization, and the unquestionable value of hematologic methods in the objective measurement of iron metabolism have been increasingly recognized. It is regretted that more extensive and accurate data on the condition of the blood of these children are not available. The results of estimations by comparison with the Tallqvist scale indicated that the hemoglobin levels of the

children were in the neighborhood of 80% throughout the study. Both of the 3-year-olds showed a slight increase in hemoglobin during the experiment and none of the children showed a decrease. The red cell counts ranged from 6.59 $\overline{\text{M}}$ to 4.88 $\overline{\text{M}}$, and decreased slightly in each child during the study. The counts were unusually high in all children at the beginning and in no case reached a figure as low as 4.65 $\overline{\text{M}}$, which is the mean value given in Guest's findings on 278 samples of blood from children $2\frac{1}{4}$ to $5\frac{1}{2}$ years old (Guest, '38). On the basis of these rather limited data, it probably could be said that the children were in at least "average" condition throughout the study as far as their blood pictures were concerned.

The t-value as determined by the Brandt equations as well as the F-values obtained by analysis of variance showed that there was no significant difference in iron storage on the two types of diets. Accordingly, the results from the entire 63-day period for each child have been considered as a single series and are given in table 2.

Iron intakes. The iron intakes of the children were probably as uniform from period to period as could be expected in this type of study. Nevertheless, the percentage of variation in intake ranged from 54% for O to 28% for K. These percentages were calculated by dividing the difference between the lowest and highest intakes by the lowest intake. Expressed in milligrams per day, the average intakes ranged from 5.38 to 5.95, with an average for all children of 5.64. The level of intake ranged from 0.22 mg. for M to 0.42 mg. for O and averaged 0.31 mg. per kilogram of body weight. This would be considered a low level of iron ingestion when it is compared with the dietary allowances recommended by those who have made earlier studies of iron balances in children. Leichsenring and Flor ('32), for example, found that children retained iron in appreciable amounts on an intake which was about 0.19 mg. per kilogram, but that they retained over twice as much of the mineral on an intake of about 0.37 mg. They added the average excretions on the two levels of intake

TABLE 2

Average iron balances, in milligrams per day, from nine 7-day periods on four children

SUBJECT	DIET	PERIOD	INTAKE		EXCRETION				RETENTION	
			Total	Per kilogram	Urine	Feces	Total	Per kilogram	Total	Per kilogram
			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
O	Fortified farina	1	7.04	0.42	0.13	4.80	4.93	0.29	2.11	0.13
		2	6.23	0.37	0.17	3.81	3.98	0.24	2.25	0.13
		3	6.13	0.36	0.14	4.46	4.60	0.27	1.53	0.09
	Plain farina	4	4.63	0.27	0.12	4.26	4.38	0.26	0.25	0.01
		5	5.23	0.31	0.24	4.11	4.35	0.26	0.88	0.05
		6	5.03	0.29	0.15	3.32	3.47	0.20	1.56	0.09
	Fortified farina	7	5.14	0.30	0.21	3.75	3.96	0.23	1.18	0.07
		8	4.57	0.27	0.19	3.59	3.78	0.22	0.79	0.05
		9	4.98	0.29	0.22	4.71	4.93	0.28	0.05	0.01
	Mean		5.44	0.32	0.17	4.09	4.26	0.25	1.18	0.07
R	Plain farina	1	5.96	0.38	0.13	4.16	4.29	0.27	1.67	0.11
		2	5.23	0.33	0.24	4.16	4.40	0.28	0.83	0.05
		3	4.98	0.31	0.14	3.82	3.96	0.25	1.02	0.06
	Fortified farina	4	5.14	0.32	0.12	3.82	3.94	0.25	1.20	0.07
		5	5.61	0.35	0.27	4.29	4.56	0.28	1.05	0.07
		6	5.96	0.37	0.12	3.64	3.76	0.23	2.20	0.14
	Plain farina	7	6.08	0.37	0.21	4.51	4.72	0.29	1.36	0.08
		8	4.47	0.27	0.22	3.43	3.65	0.22	0.82	0.05
		9	5.01	0.31	0.18	4.70	4.88	0.30	0.13	0.01
	Mean		5.38	0.33	0.18	4.06	4.24	0.26	1.14	0.07
M	Fortified farina	1	6.16	0.30	0.23	4.23	4.46	0.22	1.70	0.08
		2	5.42	0.26	0.26	4.93	5.19	0.25	0.23	0.01
		3	7.22	0.35	0.19	4.13	4.32	0.21	2.90	0.14
	Plain farina	4	5.21	0.25	0.18	3.12	3.30	0.16	1.91	0.09
		5	5.44	0.26	0.22	4.40	4.62	0.22	0.82	0.04
		6	6.47	0.30	0.36	4.13	4.49	0.21	1.98	0.09
	Fortified farina	7	5.73	0.27	0.24	4.42	4.66	0.22	1.07	0.05
		8	4.72	0.22	0.31	4.35	4.66	0.22	0.06	0.00
		9	5.80	0.27	0.23	5.00	5.23	0.24	0.57	0.03
	Mean		5.80	0.28	0.25	4.30	4.55	0.22	1.25	0.06
K	Plain farina	1	7.06	0.35	0.15	5.24	5.39	0.27	1.67	0.08
		2	6.02	0.30	0.17	5.41	5.58	0.28	0.44	0.02
		3	5.58	0.28	0.14	4.09	4.23	0.21	1.35	0.07
	Fortified farina	4	5.51	0.27	0.13	3.49	3.62	0.18	1.89	0.09
		5	6.03	0.30	0.31	5.26	5.57	0.28	0.46	0.02
		6	5.91	0.29	0.12	3.54	3.66	0.18	2.25	0.11
	Plain farina	8	5.92	0.30	0.15	3.77	3.92	0.20	2.00	0.10
		9	5.62	0.28	0.25	4.29	4.54	0.23	1.08	0.05
	Mean		5.95	0.30	0.18	4.38	4.56	0.23	1.39	0.07
O and R mean			5.41	0.33	0.18	4.07	4.25	0.26	1.16	0.07
M and K mean			5.87	0.29	0.21	4.34	4.55	0.22	1.32	0.07
All			5.64	0.31	0.20	4.20	4.40	0.24	1.24	0.07

to arrive at a "requirement" for maintenance and growth of 0.32 mg. per kilogram. Then they still further added, because of individual differences in children and because of the question of availability of iron in food, a 50% margin of safety, arriving at a standard dietary allowance of 0.48 mg. per kilogram per day. Daniels and Wright ('34) found that diets containing 0.75 mg. of iron per kilogram resulted in no higher retentions than those containing 0.59 or 0.65 mg. and concluded that approximately 0.60 mg. per kilogram would meet the needs for maintenance and growth in normal pre-school children. Both of these groups of workers have suggested

TABLE 3

Summary of iron intakes and retentions, in milligrams per kilogram of body weight, from available studies of pre-school children

AUTHORS	YEAR	NUMBER OF SUBJECTS	DAYS IN BALANCE PERIODS	INTAKE PER KILOGRAM	RETENTION PER KILOGRAM
Rose et al.	1930	1	9	0.33	—0.08
Leichsenring and Flor	1932	4	5	0.19	+0.07
		4	5	0.37	+0.18
Daniels and Wright	1934	8	5	0.63	+0.18
Ascham	1935	6	15	0.59	+0.07
This study	1940	4	63	0.31	+0.07

dietary allowances considerably above that received by the children in this study. Reference to table 3, in which data from previous studies on iron balances are briefly summarized, shows that the average level of iron ingestion in this study was about one and a half times the lowest intake reported (Leichsenring and Flor, '32) and equivalent to about half of the highest intakes (Daniels and Wright, '34).

Iron excretions. An inspection of the bottom row of figures in table 2 which represents an average of all the data collected on the four children, shows that the usual paths of excretion accounted for a loss of about 78% of the iron ingested. Urinary iron was almost negligible in quantity. For these four subjects the total output averaged only 0.20 mg. per day. Total

fecal iron averaged 4.20 mg. per day, with a fairly wide range of from 3.12 mg. to 5.41 mg. This resulted in an average total daily output of 4.40 mg. (4.20 + 0.20). From the figures on excreted iron for individual 7-day periods, however, it can be seen that there was a rather wide variation in the total quantity of iron lost from the body, the excretion of iron varying from 3.30 to 5.58 mg. per day. The percentage variation was 52% for M, 42% for O, 33% for R and 31% for K. These variations are of the same order of magnitude as those in the intake levels. When the data from all periods, however, are averaged, it is evident that the total daily output of iron varied less than 0.5 mg. among the children. Recent work on the iron metabolism of healthy adults indicates that the body has little capacity for excreting iron (Widdowson and McCance, '37). It seems that such a finding precludes the use of levels of excretion of iron as a basis for the estimation of requirement. Excretion can be considered of only secondary importance in determining needs, and fecal iron simply represents iron which, for some reason, was not absorbed.

Iron retentions. The iron retention of these children averaged 1.24 mg. per day or 0.07 mg. per kilogram. When individual 7-day balances are examined it may be observed that none of the balances were "negative," although some of the retention figures undoubtedly represent equilibrium or quantities of iron within the limits of experimental error. If a balance of 0.02 mg. per kilogram or less is considered as practical equilibrium, this state of very low or negligible retention existed during one or two 7-day periods for each of the children during the study. On the other hand, rather high retentions of 0.10 mg. or more per kilogram per day were found in an equal number of periods for all children. An average of only 22% of the total intake was stored by these growing children. Further, the retention of iron varied considerably from period to period. Of course, in some individual periods the level of retention was directly proportional to the level of intake, that is, a low storage on a slightly lowered intake or a high storage on a slightly increased intake; but

this was only true in approximately 40% of the balances. Correlation of retention with intake was more evident in subjects O and M than in R and K.

Recognizing that lack of uniformity in techniques employed in balance studies makes comparisons of any two of them difficult, it may be of interest, nevertheless, to examine the storage of iron on some levels of intake which have been reported in other studies, as summarized in table 3. If, first, storages on intakes very similar to the intakes of the children in this study are compared, it will be observed such storages vary from —0.08 mg. in the one child studied by Rose et al., to 0.18 mg. per kilogram in the four children studied by Leichsenring and Flor, the average retention of 0.07 mg. in this study representing about a mid-point between these two extremes. If next, the intakes which permitted retentions identical with the average retention in this study are compared, it is seen that these intakes varied from 0.19 mg. to 0.59 mg., the intake of this study averaging 0.31 mg. In other words, there is no consistent level of storage on a given intake. Probably previous nutritional status with regard to iron is a much more important factor in determining the absorption and utilization of iron from food than any observation which can be made during the course of a controlled balance study. It is evident from the summary of the findings of other investigators, as well as from data in this study, that growing children usually are absorbing some iron from their food for maintenance and growth.

The length of this study makes the average retention levels of somewhat more interest, perhaps, than those observed during shorter periods. Nevertheless, the interpretation of the utilization of iron in terms of the adequacy of the intake is difficult to make and possibly is not entirely justified without a more complete history of the hematopoietic processes which took place during the period of observation. One or two points, however, may be mentioned. The average level of storage of iron from the adequate and varied combination of foods in the diet was surprisingly uniform in all the

children. Within these averages, however, there were wide variations in retention, ranging from a state of practical equilibrium to levels which would be considered high. If an unbiased interpretation is made on the data of this study, an unprecedented conclusion might be reached. For instance, since the subjects seemed to be in good physical condition at the beginning and throughout the study, with blood pictures which were reasonably acceptable, and since the diet was judged adequate in other essential nutrients such as protein, calcium, phosphorus, and certain of the vitamins, it might be concluded that the children were using as much dietary iron as they needed during the course of the investigation. Certainly the wide variations in period to period retentions support such a statement. In other words, variations in retention of iron during the 2-month period reflect variations in metabolic demand, with the retention of considerable amounts of iron at times, and with almost negligible storage at other times. On the other hand, a more conservative viewpoint would still direct toward the advisability of providing liberal quantities of iron in food for children. The surprisingly uniform average storage of iron by all the children, and certain indications from the limited hematologic records, may mean that the children made as good utilization of iron as the particular diet permitted. Further, these data do not preclude the possibility that the subjects would have been in better nutritional condition with regard to iron had they had a different diet and/or a higher level of iron intake. It will probably appear to some readers, therefore, that the suggestion of the adequacy of the rather low iron intake fed the children in this study is of doubtful propriety, especially when the many hazards to which iron is subject in the course of digestion and assimilation are taken into consideration. Perhaps these findings are best used as a basis for the statement that great anxiety over the need for providing impractically high levels of iron in children's diets is unwarranted. In the future, studies of iron metabolism, particularly if directed toward the establishment of dietary allowances, should include data on

utilization of iron within the body, since accumulating evidence indicates both the efficient use of absorbed iron and the low capacity for excretion of iron. At the moment, measures of blood changes correlated with balance studies hold the greatest promise as criteria for iron utilization.

SUMMARY

Thirty-five iron balances on four normal pre-school children, 3 and 5 years old, are reported in this study. For 63 days, which were divided into nine 7-day experimental periods, the children received daily a mixed diet furnishing an average iron content of 5.64 mg., or 0.31 mg. per kilogram of body weight.

On this iron intake varying amounts of the mineral were stored, ranging from a state of practical equilibrium to 2.90 mg. of iron per day. The average iron retention by all children throughout the study was 1.24 mg., or 0.07 mg. per kilogram of body weight per day.

The average level of retention represented about 22% of the intake of iron.

The replacement of 1 ounce of plain farina in the basal diet with an equivalent quantity of farina fortified with irradiated yeast containing 120 I.U. of vitamin D had no significant effect on iron retentions.

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THE EFFECT OF DRY HEAT UPON THE ANTICATARACTOGENIC QUALITY OF CERTAIN PROTEINS [1]

MARY D. HENDERSON [2] AND HELEN S. MITCHELL

Department of Home Economics Research, Massachusetts State College, Amherst

The incidence and rate of cataract development in rats fed galactose are increased by an inadequate amount of protein and inhibited by a high protein ration. These findings were reported by Mitchell and Cook ('37, '38 a, b) and partially confirmed by Yudkin and Geer ('40). The comparative effects of different types or sources of protein in such rations were reported by Mitchell and Cook ('38 a). Lactalbumin, beef muscle, cod fish, beef fibrin, soy bean and wheat gluten, fed on an equivalent nitrogen basis, gave approximately the same degree of protection or slightly less and egg albumin somewhat more protection than casein. The differences were not great, and certainly the anticataractogenic quality did not parallel the growth value of these proteins.

Mitchell, Cook and Henderson ('40) reported work with several nitrogenous factors other than protein. Since an enzymic hydrolysate of casein gave almost the same degree of protection as the nitrogen equivalent in commercial casein, it was evident that the intact protein molecule was not an essential factor. When cystine and methionine were reinvestigated with more accurate feeding technique than in earlier work, they

[1] Contribution no. 375 of the Massachusetts Agricultural Experiment Station, from the Department of Home Economics Research.

[2] Most of the data reported in this paper were presented by Mary D. Henderson in partial fulfillment of the requirements for the degree of Master of Science, Massachusetts State College.

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showed little if any inhibitory effect. Urea and choline, representative of simpler nitrogenous compounds, gave negative results.

Another approach to the problem was the investigation of the effect of proteins modified by physical or chemical means so as to alter their nutritive values. Chick et al. ('35) reported that casein suffered a marked loss in biologic value due to prolonged exposure to dry heat. Morgan et al. ('30, '31) had previously shown that less drastic heating lessened the growth value of both cereal proteins and casein. Thus the question arises: does the type of treatment which affects the growth value of protein also affect its cataract-inhibiting quality?

The data presented in this paper show the effect of prolonged exposure to dry heat upon the growth value and anti-cataractogenic quality of four proteins—casein, egg albumin, wheat gluten and beef fibrin—when incorporated into rations containing galactose.

EXPERIMENTAL PROCEDURE

The four proteins used were chosen as representative of distinct types and were easily available from commercial sources. A drastic heat treatment which might be expected to alter the growth value of the protein was obviously necessary in order to answer the question raised, and yet it was essential not to sacrifice edibility if the experiment were to be successful. In the present series of tests the raw dried commercial egg albumin was fed both as purchased and in the coagulated form previously used in this laboratory. The latter was prepared by dissolving the raw product in a large volume of warm water and coagulating with live steam. The filtered coagulum was spread on trays, dried at low temperature in circulating air and ground to a coarse powder. The proteins were heated as follows: Weighed amounts were spread in 1-inch layers in glass trays and heated for 96 hours in an electric oven at 125°C. Each batch was thoroughly stirred at 24-hour intervals. Each lot of protein was sufficient for the entire experiment and was divided into two portions—one unheated and

the other heated. Nitrogen determinations on each form served as the basis for calculating the protein content as shown in table 1.

The formulas for the experimental rations were calculated to provide 15% of protein. Cornstarch was added in different amounts according to the amount of the crude protein used. The skeleton formula for the galactose ration had the following percentage composition: galactose, 25; protein 15 (actual amount of crude protein calculated from figures in table 1); hydrogenated cottonseed oil,[3] 9; cod liver oil, 2; salt mixture no. 351,[4] 2.5; brewers' yeast, 5, and cornstarch to make up to 100%.

TABLE 1

PROTEIN	COMMERCIAL OR UNHEATED FORM			HEATED 96 HOURS AT 125°C.		
	Nitrogen	Conversion factor	Protein	Nitrogen	Conversion factor	Protein
Casein	14.25	× 6.38	– 90.9	14.92	× 6.38	– 95.17
Egg albumin, raw	13.25	× 6.38	– 84.5			
Egg albumin, coagulated	14.04	× 6.38	– 89.6	14.75	× 6.38	– 94.0
Wheat gluten	14.29	× 5.7	– 81.46	15.05	× 5.7	– 85.95
Beef fibrin	14.55	× 6.25	– 90.95	15.15	× 6.25	– 94.71

A second series of rations was made like the first in all respects except that the galactose was omitted and all the carbohydrate was furnished by cornstarch. The rats fed the galactose-free diets served as controls with respect to the effect of heat upon the growth value of the proteins when cataract formation was not a factor.

The experiment was planned to make the following comparisons possible: (1) the effect of dry heat treatment upon the growth value of proteins fed in a ration containing starch as the source of carbohydrate (table 2); and (2) the effect of dry heat treatment upon the growth and anticataractogenic values of proteins fed in a ration containing 25% of galactose comparing, first, the plain vs. heated protein (table 3, part 1)

[3] Crisco.
[4] Hubbell, R., L. B. Mendel and A. J. Wakeman, 1937, J. Nutrition, vol. 14, p. 273.

and, second, three unheated proteins and three heated proteins (table 3, part 2).

The "paired" feeding method, used throughout this experiment, was necessary not only to insure a comparable intake of protein, but also an equal intake of the cataract-producing agent, galactose. In previous work it has been noted that a lower food consumption may greatly delay the onset of cataract because of the reduced intake of galactose, and not because of any protective nature of the ration.

The variation in litter susceptibility to cataract was also a factor in planning experiments. Litter mates of the same sex, as far as possible, were grouped for paired feeding, and at least three litters of rats were used in making each comparison. Rats from a susceptible strain were used throughout, and were started on the experimental rations at 25 or 26 days of age.

A weekly ophthalmoscopic examination of the eyes was made. A drop of a 0.2% solution of atropine sulphate, administered about 15 minutes beforehand, served to dilate the pupils completely. The slight changes thus recorded served as a warning of more advanced changes or, in some cases, became the only criterion for judging the extent of the damage to the lens when mature cataract failed to develop. The first appearance of a definite posterior opacity visible to the naked eye was recorded as "mature posterior" and the number of days on the experimental ration until this type of opacity appeared has been used as the time figure for measuring the rate of cataract development. Failure to show visible opacities at the end of the experimental period of 8 or 10 weeks was recorded as "immature," even though advanced changes visible with the ophthalmoscope had been present for many weeks in most cases. This procedure is explained in detail because different laboratories are using various standards for the development of cataract, and this fact may lead to some confusion unless resulted are carefully interpreted.

Blood sugar determinations, using the Benedict micro method, were made on most of the rats during the period of cataract development and upon control animals during the

same period. From one to three observations were made on each rat and averaged as representative of that animal. A high blood galactose superimposed on a relatively normal amount of glucose was regularly found in rats on galactose rations. It seemed desirable to determine whether there might be any significant alteration in the total blood sugar level with the various forms of protein, especially in those cases where there appeared to be a difference in the protective action against damage to the lens.

TABLE 2

The effect of dry heat upon the growth value of proteins when fed in a starch ration

PAIRED FEEDING OF RATIONS CONTAINING 15% OF PROTEIN AS		NUMBER OF RATS	AVERAGE GROWTH IN 3 WEEKS	AVERAGE FOOD INTAKE IN 3 WEEKS	AVERAGE BLOOD SUGAR
			gm.	*gm.*	*mg./100 cc.*
Casein	%				
plain	16.5	3	42	181	111
heated	15.8	3	24	181	115
Egg albumin					
raw, dried	17.7	2	35	157	128
coagulated	16.7	2	30	157	124
heated	16.0	2	34	157	129
Wheat gluten					
raw	16.4	3	22	147	126
heated	16.0	3	6	147	133
Beef fibrin					
raw	16.5	4	74	201	124
heated	15.8	4	63	201	128

RESULTS AND DISCUSSION

The data presented here confirm those from other laboratories concerning the loss of growth value of certain proteins when subjected to long exposure to dry heat. This loss of biologic value was evident to about the same degree with the starch rations (table 2) as with the galactose rations (table 3). The degree of heat injury to the proteins, calculated from the retardation of growth observed, was in the decreasing order, gluten, casein, fibrin, egg albumin, with the last showing little if any deterioration.

The question as to whether the order of loss in protective action against cataract would correlate with the loss in growth value is quite definitely answered in the negative. A careful study of table 3, which summarizes all results on the galactose rations, reveals ample evidence that the loss of protective action does not correlate with either the loss of growth value or with food consumption.

TABLE 3

The effect of dry heat upon the growth and anticataractogenic value of proteins when fed in a galactose ration

Paired feeding of rations containing 25% galactose plus 15% of protein as		Number of rats	Growth 3 weeks grams	Food eaten 3 weeks grams	Blood sugar mg. per 100cc.	Incidence of mature cataract percent	Cataract development Time on experimental ration before opacities are visible days
Casein	percent						
plain	16.5	6	46	188	225	92	36.0
heated	15.8	10	29	187	286	100	18.5
Egg albumin							
raw dried	17.7	9	46	206	240	78	43.3
coagulated	16.7	9	48	208	256	83	40.0
coag. heated	16.0	10	40	212	272	95	28.2
Wheat gluten							
raw	16.4	9	22	151	240	83	33.2
heated	16.0	10	9	151	244	95	29.4
Beef fibrin							
raw dried	16.5	12	66	201	–	83	38.0
heated	15.8	12	52	201	–	87	35.8
Untreated proteins compared:							
Casein	16.5	6	49	209	255	75	39.7
Egg albumin (coagulated)	16.7	6	52	209	242	83	38.4
Wheat gluten	16.4	6	45	209	232	75	37.0
Heated proteins compared:							
Casein	15.8	6	18	179	274	83	27.3
Egg albumin	16.0	6	22	179	273	92	27.2
Wheat gluten	16.0	6	16	179	224	67	41.7

In the first four groups in table 3 the results with heated and untreated proteins are compared, the solid bar representing the rate of cataract development on the heated protein in each case. The wheat gluten which lost the most in growth value upon heating, lost almost none of its protective action. On the other hand, egg albumin, which lost the least in growth value suffered a definite loss in protective action. Heated casein lost in both values while heated fibrin lost almost nothing in protective value and very little in growth value.

The last two groups in table 3 make possible a comparison among three of the untreated proteins and among the same three when heated. The data in the last group tend to confirm the previous conclusion that wheat gluten loses less in anti-cataractogenic value when heated than do casein and egg albumin. The lower food consumption of the heated protein rations as compared with that of the untreated protein rations adds significance to the already obvious differences. In spite of the reduced intake of galactose, two of the heated proteins allowed an increase in the rate of cataract development.

The blood sugar values of all rats on galactose rations (table 3) are pathologic, as is to be expected, and contrast sharply with those of rats on starch rations (table 2). The relatively higher values in both groups of rats fed heated casein or heated egg albumin, as compared with litter mates fed the respective untreated proteins, are suggestive of an explanation for the more rapid lenticular injuries in the former. However, the variability of the blood sugar values for several rats on the same ration and for the same rat at different times raises some doubt as to the significance of the average figures presented. Studied statistically, the differences between the plain and heated casein groups are significant, but between the raw and heated egg albumen they are questionable. Every effort was made to keep the conditions constant as to the amount of food consumed and the interval of time before the blood was drawn, but at best the sample does not always represent the maximum rise in blood sugar during absorption.

The influence of these different protein preparations upon the metabolism of galactose as measured by the level of the sugar in the blood is worthy of further consideration. In our earlier work with different quantities of casein in the ration, differences of the same order were noted. When the protein was increased from 15 to 45% the blood sugar average dropped from 276 to 213 mg. per 100 cc. of blood. At the same time the low protein (5%) group had almost exactly the same blood sugar as the medium protein (15%) group, and yet the former

developed cataract much more rapidly. Thus while the differences in blood sugar which do exist are in the right direction, yet they scarcely seem sufficient to explain the retardation or hastening of the pathologic changes in the lenses. Perhaps the lens of the young rat is particularly susceptible to damage and sensitive to slight changes in the blood sugar level. It may be the extent of fluctuation in the level of blood sugar which is injurious rather than the absolute level reached after a given amount of a ration containing galactose has been consumed.

In previous publications from this laboratory it has been suggested that the galactose in the blood stream may cause direct damage to the lens capsule, altering its permeability and thus allowing a shift in the ionic equilibrium which maintains the proteins in the lens in perfect transparent solution. The observed protective action of protein must be exerted either by lowering the level of the injurious factor in the blood stream or by increasing the resistance of the capsular membrane to injury. The failure to observe significant differences in the blood sugar level between different ration groups which did show a striking difference in the degree of lenticular injury has led us to favor the hypothesis that the protective action of the protein, in some way, concerns the lens or its capsular membrane. The degree of galactemia is undoubtedly a contributing factor. Thus, if some nitrogenous factor does influence galactose metabolism, by increasing the rate of glycogenesis in the liver, for instance, this point is worthy of further study. If the protein factor is protective in the presence of the injurious agent, galactose, it is possible that the same factor might exert a similar protection against other injurious agents in the blood stream. The nature of the protective factor in protein and its mode of action in the presence of a galactemia are still under investigation.

SUMMARY

1. Since protein appears to exert a protective action against galactose cataract in rats, the question has been raised as to whether the type of treatment which is known to decrease

the growth value of a protein will also affect its cataract-inhibiting quality.

2. Four proteins, casein, egg albumin, wheat gluten and beef fibrin, were fed as purchased and after heating for 96 hours at 125°C. Each protein was incorporated into a 25% galactose ration at a level to provide 15% of protein.

3. The growth value of these proteins was damaged by heat in the decreasing order: gluten, casein, fibrin, egg albumin.

4. The anticataractogenic property of these proteins was diminished by heating in the decreasing order: casein, egg albumin, gluten, fibrin.

5. Since the losses in the two properties of protein due to exposure to heat do not run parallel, it follows that the amino acid or group of amino acids which exert the protective action are not the same as those essential for growth.

6. In some instances the blood sugar values correlated with the more rapid cataract development but there is considerable doubt as to whether the degree of galactemia alone can account for the differences in lenticular injury.

7. It is possible that the protein factor may be protective by some local action in the eye rather than by influencing galactose metabolism.

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THE EFFECT OF THE HYDROLYTIC PRODUCTS OF CASEIN AND DEAMINIZED CASEIN ON THE CATARACTOGENIC ACTION OF GALACTOSE [1]

EDWIN L. MOORE,[2] MARY D. HENDERSON, HELEN S. MITCHELL
AND WALTER S. RITCHIE

*Departments of Chemistry and Home Economics Research,
Massachusetts State College, Amherst*

Earlier investigations of the influence of the type and amount of protein and other nitrogenous factors on the cataract-producing action of galactose have been reviewed in a previous paper (Henderson and Mitchell, '41). Pursuant to the theory that a type of treatment which alters the growth value of a protein may or may not affect its anticataractogenic quality, casein deaminized by treatment with nitrous acid has been the subject of investigation. The inadequacy of deaminized casein as a source of protein, shown by Hogan and Ritchie ('34), was manifested by the anemic condition of their rats as well as by retarded growth and early death. These results seemed to indicate that during deaminization either an essential nitrogenous factor is removed, or a toxic substance is produced.

In preliminary experiments in this laboratory the fact was verified that rats could not survive when deaminized casein was the sole source of protein in the ration; when no more than two-thirds of the protein was supplied by deaminized casein

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and the other third by commercial casein, growth was subnormal but most of the rats survived. When this combination of proteins was incorporated into a ration which contained 25% of galactose it was evident that the nitrous acid treatment had rendered the casein more protective against cataract although reducing the growth value. This finding was quite contrary to what one would expect considering the supposed loss of nutritive elements. The unpalatability of rations containing the deaminized casein made it necessary to use paired feeding in comparing the effect of the plain and treated casein. Although this procedure, of necessity, reduced the consumption of galactose and the consequent rate of cataract development in both groups, the protective action of the deaminized casein was evident, especially in the more susceptible litters.

These findings seemed to warrant some further investigation of deaminized casein and its hydrolytic products, in the hope of finding a fraction which might contain the protective factor or factors. It seemed possible that perhaps some new compound is formed in the process of deaminization, which is responsible for the increased protection observed.

The plan of the present investigation was to hydrolyze, by both acid and enzymic hydrolysis, large batches of commercial casein and deaminized casein and then fractionate the hydrolysates into the major amino acid groups. It was necessary to work with quantities sufficient to permit the feeding of the fractions to experimental animals. The preparation of the deaminized casein, hydrolysates and fractions was carried out as quantitatively as possible, with careful records of all yields and analyses of the various products for nitrogen. The formulas for the galactose rations containing the various preparations were calculated according to the yield and nitrogen content as will be explained later.

CHEMICAL PREPARATIONS FROM CASEIN

1. Deaminized casein. The procedure as outlined by Dunn and Lewis ('21) was followed. Yields averaged about 90% and were of the order obtained by the original investigators.

Analyses of the original casein and the deaminized product showed 14.04 and 14.21% nitrogen, 2.58 and 1.69% ash, and 7.46 and 4.21% moisture respectively.

2. *Acid hydrolysate and fractions.* In the preparation of the acid hydrolysate and its fractions the method of Dakin ('18) was employed and batches of 500 gm. each of casein and deaminized casein were used. Table 1 gives the hydrolytic

TABLE 1

Summary of casein and deaminized casein hydrolysis. Figures represent the per cent of the original nitrogen recovered. Letters are abbreviations used for subsequent reference to products prepared and used in feeding tests

INITIAL PRODUCT	TOTAL HYDROL-YSATE	FRACTIONATED HYDROLYSATES					
		Humin	Barium sulfate precipi-tate	In-soluble solids	Monoamino acids	Diamino and dicarb-oxylic acids	Proline and peptides
Acid hydrolysis							
Casein C	75.45 CAH	0.19	9.83		24.86 CAHMA	25.58 CAHDD	15.50 CAHPP
Deaminized casein DC	83.82 DCAH	0.58	9.39		18.70 DCAHMA	39.04 DCAHDD	14.95 DCAHPP
Enzymic hydrolysis							
Casein C	93.93 [1] CEH			4.93	21.81 CEHMA	48.73 CEHDD	—[2] CEHPP
Deaminized casein DC	90.56 [1] DCEH			4.67	10.35 DCEHMA	57.12 DCEHDD	—[2] DCEHPP

[1] Nitrogen of the trypsin is not included in the figure for the total hydrolysate but could not be excluded from the figures for the fractions.

[2] A portion of this fraction was lost and therefore the per cent recovered could not be calculated.

products prepared, the abbreviations used and the percentage of the total nitrogen recovered in each fraction. The hydroly-sates and fractions, other than the monoamino acid fractions, were dried on cornstarch because of their sticky consistency.

3. *Enzymic hydrolysate and fractions.* It is well known that acid hydrolysis is, in general, more destructive than enzymic hydrolysis. The latter process more nearly simulates the breakdown of protein in the animal body. For these reasons,

casein and deaminized casein were subjected to tryptic hy-
drolysis under controlled conditions as follows: 350 gm. of
casein (or deaminized casein) were suspended in 3.5 liters
of distilled water. To this suspension were added 28 gm. of
anhydrous sodium carbonate (to produce a pH of about 8.1),
17.5 gm. of trypsin [3] and a few crystals of thymol as preserva-
tive. A thin layer of toluene was added and the flasks stop-
pered with cotton and incubated at about 37°C. for 3 days. On
the third day another 17.5 gm. of trypsin were added to each
flask and the pH again adjusted to 8.1. This adjustment was
made every 3 days throughout the digestion period of 3 weeks.
Finally the digestion mixture was boiled to drive off the toluene
and to stop any further action of the enzyme. The mixture
was then concentrated to a thick syrup in a distilling flask
under reduced pressure and deposited on cornstarch and dried.

Using another 350 gm. each of casein and deaminized casein,
the same procedure was followed to the point where the hy-
drolysate was boiled to free it from toluene. At this point the
digest was filtered and the residue thoroughly washed to free
it from soluble material. This residue was dried on cornstarch.
The filtrate and washings were concentrated on the steam bath
and were subjected to fractionation by Dakin's method. The
fractions were isolated and concentrated by the same proce-
dure as in the acid hydrolysis.

All fractions which were deposited on starch were dried in
an oven at approximately 40°C. with continuous circulation of
air. The resulting material was ground to a powder in a ball
mill. Representative samples of each dry powder were ana-
lyzed for nitrogen in order to calculate the total nitrogen re-
covered in each fraction and the proportion of each fraction
to use in the feeding trials. Blanks were subtracted for the
cornstarch used in all preparations and for the trypsin used
in the total enzymic hydrolysate. It was impossible to make
correction for the trypsin in the fractions, because the per-
centage of trypsin remaining in each fraction could not be
determined.

[3] The trypsin was obtained from Fairchild Bros. and Foster, New York, N. Y.

Comments on relative yields. A larger percentage yield of monoamino acids was obtained from casein than from deaminized casein by both methods of hydrolysis. This result was unexpected because the lysine, after deaminization of the casein, should shift from the diamino to the monoamino acid fraction if the theories as to the alteration in the molecule are correct. This finding points toward an incomplete hydrolysis of the deaminized product to the free amino acids. The relatively larger yield of diamino and dicarboxylic acids from the deaminized casein supports this theory because any soluble material not extracted in the preceding groups would appear in this fraction. The loss of nitrogen in the barium sulfate precipitate of the acid hydrolysis does not occur in the enzymic hydrolysate and thus more nearly the full value of the protein should be recovered from the latter.

EXPERIMENTAL FEEDING OF CASEIN PREPARATIONS

The ration containing 25% of galactose as the cataractogenic agent was modified with respect to the protein and cornstarch only and had the following percentage composition: casein (commercial), 5.5; galactose, 25; hydrogenated cottonseed oil,[4] 9; cod liver oil, 2; salt mixture,[5] 2.5; brewers' yeast, 5; casein, deaminized, hydrolysates or fractions, 10 (calculated according to nitrogen content or equivalent fraction); cornstarch—to make total up to 100%.

The amount of the total hydrolysate incorporated in the ration was calculated on the basis of the nitrogen equivalent of the 10% of protein fed as casein. The amount of any fraction fed, replacing the 10% of protein, was proportional to the percentage yield of that fraction from the total casein or deaminized casein hydrolysates in terms of the nitrogen content. This procedure was adopted in order that any fraction might afford as much protection but no more than its 10% protein control, because the amount of each fraction used should theo-

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[4] Crisco.

[5] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 J. Nutrition, vol. 14, p. 273.

retically be the same in the two rations. A fraction which carried no protective action would add nothing to the degree of protection afforded by the 5.5% of casein already present in the basal ration. So far as possible the corresponding fractions prepared from the acid and enzymic hydrolysis differed only with respect to the nitrogen content of the enzyme used.

All rats used in this experiment were from one strain known to be susceptible to galactose cataract. Paired litter mates were used throughout and were started on experimental rations at 25 or 26 days of age. The number of days on experimental rations until visible opacities appeared were recorded as the time for the development of cataract. Failure to show visible opacities at the end of the experimental period of 8 weeks was recorded as "immature" with the degree of early lenticular damage estimated by ophthalmoscopic examination. A limited number of rats fed under rigidly controlled conditions was necessitated by the nature of the experiment and the small amounts of the fractions available.

The equalization of food intake between pairs or triplets insured a comparable intake of the casein preparation being tested and also an equal intake of the cataractogenic agent, galactose. Thus, the only valid basis for interpreting the data reported in table 2 is to compare the incidence and rate of cataract development in litter mate controls eating the same amount of food. The poor growth of all rats on deaminized casein products was due to a distaste for these products. Deaths due to inanition in the DCAH and DCAHDD groups made complete observations impossible. In general it was noted that the enzymic hydrolysate and its fractions were less objectionable to the rats, judged by the growth and food intake figures. Thus the results with enzymic hydrolytic products were more satisfactory than those with the acid products..

The results of the feeding tests with all of the casein preparations are summarized in table 2. Growth and food intake figures are significant to the extent that the low intakes retarded both growth and cataract development. In studying

TABLE 2

Data on rats fed casein and deaminized casein and their hydrolytic products incorporated in a galactose ration. Paired feeding with controls on casein (C) and deaminized casein (DC)

CASEIN OR ITS DERIVATIVES IN THE RATION [1]	NUMBER OF RATS	AVERAGE GROWTH IN 3 WEEKS	AVERAGE FOOD EATEN IN 3 WEEKS	MATURE CATARACT		BLOOD SUGAR
				Incidence	On exp. ration	
		gm.	gm.	%	days	mg./100 cc.
DC	16	15	106	*47*	*59.6*	225
C	10	17	106	*60*	*46.5*	233
CAH	4	—3	87	100	36.3	—
C	2	18	89	50	47.0	—
CAHMA	3	42	191	100	23.6	220
C	3	60	194	100	26.5	245
CAHDD	3	6	135	100	28.5	—
C	2	17	135	67	48.3	—
CAHPP	3	8	138	100	29.3	246
C	2	32	139	0	56.0	283
DCAH	4	All died in 9–14 days				
DC	4	All died or were moribund due to starvation				
DCAHMA	4	9	111	75	33.8	237
DC	4	4	111	50	50.0	238
DCAHDD	4	—12	89	Died due to starvation		
DC	4	0	91	Died due to starvation		
DCAHPP	4	0	97	75	36.2	253
DC	4	5	97	25	52.6	247
CEH	4	60	209	75	39.5	217
C	4	65	209	25	49.6	213
CEHMA	4	46	216	100	17.8	262
C	2	63	216	100	28.0	243
CEHDD	3	30	161	100	21.7	248
C	2	37	161	66	46.7	229
CEHPP	2	12	140	100	15.5	227
C	2	31	143	75	35.5	271
DCEH	6	10	105	*25*	*54.3*	234
DC	5	9	104	*25*	*49.8*	232
DCEHMA	5	12	113	60	35.7	227
DC	4	18	113	20	54.6	223
DCEHDD	4	5	109	*25*	*50.8*	241
DC	3	6	109	*63*	*54.5*	228
DCEHPP	2	10	122	100	19.5	239
DC	2	13	122	100	36.0	271

[1] The explanation of the letter abbreviations for casein derivatives in the rations is given in table 1.

the figures on incidence and time of cataract development it will be noted that the incidence is higher and the rate more rapid than in the controls for all preparations tested except in the groups italicized. The greater protective action of deaminized casein as compared with regular casein is evident from the first set of figures. Of the hydrolytic products only the DCEH and the DCEHDD showed a protective action equivalent to or greater than the controls. This might indicate that the protective factor present in the DCEH was concentrated in the DCEHDD fraction. There was complete lack of protective action shown by most of the other derivatives or fractions. The few instances in table 2 where the incidence of mature cataract in control groups was very low or zero were due to reduced food intake or to more resistant litters. The experimental group in each of these comparisons (CAHPP and CEH) showed a high incidence and rapid development of cataract, thus supporting the conclusion that the derivative in question was not as protective as the control. The nature and mode of action of the protective factor in deaminized casein are being further investigated.

Blood sugar determinations are significant in a study of this kind because it is desirable to know whether the protective action is exerted in the presence of a high blood sugar, or whether it works by lowering the level of sugar in the blood. These determinations were made two or three times on each rat except where a moribund condition or emaciation prevented. In general the blood sugars remained high, i.e., well above 200 mg. per 100 cc. of blood, and did not vary consistently with the rate of cataract development in comparable groups. From these limited observations it would appear that the protective agent acts in the presence of the high blood sugar rather than by influencing the metabolism of the sugar directly. This local action upon the lens or lens capsule has not been explained. Factors bearing on this point have been discussed elsewhere (Henderson and Mitchell, '41).

SUMMARY

1. Preliminary work showed that deaminized casein was more protective against galactose cataract than the commercial casein from which it was prepared.

2. Hydrolytic products of casein and deaminized casein were prepared by both acid and enzymic hydrolysis and fractionated according to Dakin's method.

3. The enzymic hydrolysate of deaminized casein was somewhat more protective than the deaminized casein from which it was prepared, when fed as two-thirds of the nitrogen of the ration.

4. Of the fractions, the diamino dicarboxylic acids afforded as much protection as the whole hydrolysate, while the monoamino and proline and peptide fractions showed no protection whatever.

5. Blood sugar determinations indicated that the protective action was exerted in the presence of the high blood sugar and not by a lowering of the blood sugar level.

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CAROTENE AND VITAMIN A IN CATTLE BLOOD PLASMA WITH OBSERVATIONS ON REPRODUCTIVE PERFORMANCE AT RESTRICTED LEVELS OF CAROTENE INTAKE[1]

RUSSELL E. DAVIS AND LOUIS L. MADSEN

*Animal Nutrition Division, Bureau of Animal Industry,
United States Department of Agriculture, Washington, D. C.*

ONE FIGURE

According to Palmer ('22), Krukenberg (1885) deserves the credit for the first extensive study of the chromolipid pigments in the blood serum of cattle. The discovery that carotene was the precursor of vitamin A in the animal body has led to renewed interest in this field. Carotene isolated from cattle serum by Euler and Virgin ('32) was shown to have vitamin A activity and these authors cite a case where, after winter feeding of an ox, the amount in the serum was greatly reduced. Fine ('33) also fed ox serum to young rats on an otherwise vitamin A deficient diet and concluded that serum contained vitamin A. Chevallier and Choron ('35) made extracts from the blood of rats, guinea pigs, rabbits, swine, sheep, horses, cattle, and humans, and on spectrographic analyses obtained absorption bands characteristic of vitamin A. Numerous investigators in recent years have reported studies on the vitamin A and carotene contents of human plasma both in health and disease. The work of Clausen and McCoord ('38) in which they have used a modification of the Carr-Price antimony trichloride method for the determination of vitamin A is especially notable. European medical investigators have published

[1] Presented at the meetings of the American Chemical Society, Cincinnati, Ohio, April 8–12, 1940.

considerable material in this field. For example, the work of Wolff ('36) and Menken ('32) showed that people living at the lower economic levels had the lowest blood vitamin A and carotene while Sie ('37) found that patients with eye symptoms due to vitamin A deficiency also had a low blood vitamin A content. Similar findings were reported by de Haas and Meulemans ('38) who found that children with xerophthalmia had low blood vitamin A and that the blood levels could be increased by vitamin A supplements.

Moore ('39 a, b) and Flora and co-workers ('39) have made determinations of carotene in the blood of dairy cows and calves fed various levels of this substance including the use of carotene from several sources but these investigators have not reported work on the vitamin A content of blood. Studies have been reported by Madsen and Davis ('38, '39) on the spectrophotometric examination of cattle blood for both carotene and vitamin A with the view of using the procedure in the establishment of the minimum and optimum carotene requirements for various physiological functions and for diagnosis of vitamin A deficiency or degree of depletion in cattle. In these studies it was shown that determinations of carotene and relative vitamin A values could be made on extracts prepared from blood plasma. The present paper reports additional studies on methods used and the relation of carotene intake to the carotene and vitamin A content of cattle blood plasma, the relation of the carotene content to the vitamin A content of plasma, together with initial plasma values and observations on the reproductive performance of cows at restricted levels of carotene intake.

EXPERIMENTAL

Preparation of blood plasma sample

Venous blood was drawn from the jugular vein in the morning before feeding. Lithium oxalate (1.5 to 2 mg. per milliliter of blood) was used as the anticoagulant and plasma was separated by centrifuging. Ten to 50 ml. of plasma or serum are required for satisfactory spectrographic determinations of

carotene and vitamin A, depending on the amount present in the sample. However, satisfactory carotene determinations may be made on smaller quantities of plasma from cows receiving adequate carotene. In the use of a 50 ml. sample, the plasma was pipetted into a mixture of freshly prepared alcoholic potassium hydroxide solution made up in the proportions of 75 ml. aldehyde-free absolute ethyl alcohol and 25 ml. of 25% potassium hydroxide. In smaller samples of plasma the amount of alcoholic potassium hydroxide used may be proportionally less. The mixture was then heated on a steam bath in a stream of carbon dioxide for 30 minutes. The digest was cooled and washed into a separatory funnel and extracted by shaking with successive portions of purified [2] petroleum ether (B.P. 35–45°C.). Three or four extractions were usually sufficient. For the large samples 50 ml. of ether for the first extraction and 15 to 25 ml. portions for succeeding extractions were used. The combined ether extracts were washed with water and twice with dilute potassium hydroxide. The water and water-potassium hydroxide layers were discarded when the separation was complete. The extract was then finally washed with water until free from turbidity and potassium hydroxide. The preparation of samples for vitamin A and carotene determinations is the same to this point. Aliquot portions of extract were taken for carotene and vitamin A determinations or separate portions of plasma were extracted for each.

In preparing the extract or an aliquot portion of it for carotene analysis it was further washed with four 25 ml. portions of 92% methyl alcohol after the principle of the Willstätter and Stoll procedure for carotene, dried over anhydrous sodium sulfate, filtered and evaporated to near dryness under reduced pressure in an atmosphere of carbon dioxide. The residue was made up to volume (depending on the concentra-

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[2] The ether is treated several times with concentrated sulfuric acid until there is practically no change in color of the acid after standing in contact with the ether for several hours. The ether is then washed with water, treated with potassium permanganate crystals and dried with anhydrous calcium chloride and then distilled through a fractionating column.

tion of carotene) in a mixture of equal parts higher boiling (90 to 98°C.) purified petroleum ether and aldehyde-free absolute ethyl alcohol.

The carotene concentration of the extract was then estimated in a Duboscq colorimeter supplied with a type H4, 100 watt high intensity mercury vapor lamp in connection with a blue filter of high transmission in the region of 4500 Å and using 0.1% potassium dichromate solution as a standard. From the colorimeter readings, sector settings and cell depth for the spectrophotometric examination of the sample were estimated from a curve which was obtained by plotting densities of β carotene solutions determined spectrophotometrically as ordinates against colorimeter readings as abscissas.

Colorimeter readings give only maximum values since no information is given concerning the shape of the absorption curve. Spectrophotometric examination provides data by which the ratios between extinction coefficients at different wave lengths are calculated from which the amount and purity of the carotene in the sample are estimated. A large Littrow spectrograph with a sector photometer attachment is used for this work in this laboratory.

The light source used for the spectrophotometric determination of carotene is a 6 to 8 volt, 32-32 candle-power, automobile headlight bulb, carrying a current of 8.5 amperes. It has been found that this light source is superior to the high-speed steel electrodes used in previous studies because it yields a continuous spectrum in the region where the steel electrode spectrum has only a few lines. The headlight bulb has the additional advantage of running silently during the exposure.

The portion of ether extract of blood plasma used for the determination of vitamin A was dried over anhydrous sodium sulfate after washing with dilute potassium hydroxide and water as previously described, then filtered and evaporated to a small volume (5 to 10 ml.) under reduced pressure in a stream of carbon dioxide. The extract was then transferred to a 25 ml. glass-stoppered flask for final evaporation of the solvent using the same precautions to reduce oxidation of the

vitamin A. Ten milliliters of absolute methyl alcohol was added to the residue. The mixture was shaken and placed in a bath of solid carbon dioxide overnight. Material which precipitated out on cooling was filtered off in a cooled sintered glass funnel and the absorption characteristics of the filtrate were determined spectrophotometrically. Material which was soluble in the cold methyl alcohol and had an absorption maximum at 3280 Å was taken as a measure of the vitamin A in the extract. This method does not give an absolute value for vitamin A since there are other compounds in the extract which affect the nature of the absorption in the vitamin A region. However, such interfering substances do not apparently preclude comparative results.

The source of light for the spectrophotometric determination of vitamin A used in these studies has been either a spark between high-speed steel electrodes or a low-voltage water-cooled hydrogen arc made with a quartz window as described by Allen and Franklin ('39). Curves showing the nature of the absorption spectrum of carotene and vitamin A containing extracts of blood prepared by these methods have been published by Madsen and Davis ('38).

As a general precaution in this work it is desirable to use all glass apparatus with ground-glass stoppers and stopcocks since considerable material may be dissolved from rubber tubing, either cork or rubber stoppers, and some kinds of stopcock grease which interferes with the determinations of vitamin A by the spectrographic procedure. Special care was taken to purify all reagents used and to carry the determinations through as rapidly as possible.

Cattle experiments

Hereford and Shorthorn heifer calves, born at pasture in the spring of 1937, were taken from pasture in the fall, when 6 to 8 months of age, and maintained on a low-carotenoid ration. The constituents of the ration were beet-pulp, white corn, linseed meal, soybean meal, oat straw, salt, and bone-meal, with viosterol in winter. A limited amount of alfalfa hay

was fed for about 2 months until the animals ate the grain ration readily. All but one of these animals were purchased so only approximate ages can be given. After the alfalfa. hay was discontinued all the animals developed early symptoms of vitamin A deficiency as determined by ophthalmoscopic examination of the eyes and clinical symptoms such as diarrhea, refusing food, excessive lacrimation, and nightblindness within 3 to 4 months. When symptoms were evident, the animals were given alfalfa leaf meal of known carotene content at several levels of carotene intake, furnishing 30, 45, 60, and 120 micrograms of carotene daily per kilogram of body weight. Blood plasma samples were drawn from these heifers when they were showing symptoms of vitamin A deficiency. Additional plasma analyses were made after the heifers had already reached their maximum concentration of carotene and vitamin A after several months on their respective levels of carotene intake. Blood plasma samples were also analyzed from beef cattle on pasture for comparison with plasma analyses from the animals on restricted levels of carotene intake.

As soon as possible after the heifers had been on their respective levels of carotene intake for a period of 6 months with a previous history of vitamin A depletion, they were bred by a normal bull. Blood plasma samples were again analyzed from the pregnant heifers several days before the calves were born.

RESULTS

Heifers depleted on the low-carotenoid ration and showing early symptoms of vitamin A deficiency all had very low plasma carotene and vitamin A values, while animals on the 30, 45, 60, and 120 µg. of carotene per kilogram of body weight had blood plasma values that were roughly proportional to the carotene intake as is shown in table 1. Determinations made on the blood plasma of normal Shorthorn cows on pasture showed that they had considerably higher carotene and vitamin A values but the vitamin A content of their blood tended to reach a maximum and did not increase proportionally to the carotene concentration. This is illustrated with

additional data in figure 1. The plotted points to which the curve is fitted represent average values showing the relationship of carotene to vitamin A in cattle blood plasma for a total of 107 determinations from forty cattle made on plasma from vitamin A deficient animals, the same animals on the controlled

TABLE 1

Relation of carotene intake to the level of carotene and vitamin A in cattle blood plasma

CAROTENE INTAKE IN μG./KG.	NUMBER OF		CAROTENE IN μG./100 ML.		VITAMIN A IN μG./100 ML.	
	Animals	Analyses	Range	Average	Range	Average
Low[1]	6	14	6–24	14	3–16	10
30	2	8	34–58	46	14–21	18
45	1	4	36–43	41	16–23	19
60	2	5	62–96	81	20–31	26
120	1	2	96–110	103	33–36	35
High[2]	11	11	560–1362	813	40–65	49

[1] Animals depleted on low-carotenoid diet and showing symptoms of vitamin A deficiency.

[2] Animals on pasture.
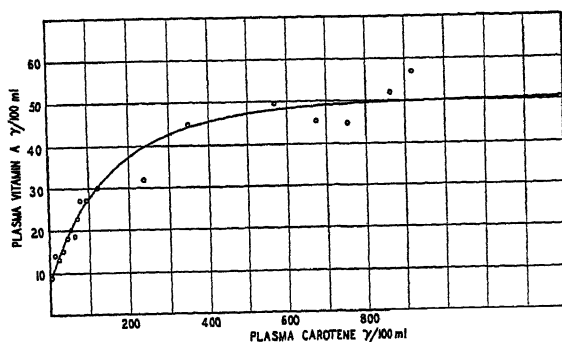
Fig. 1   Micrograms of carotene and vitamin A per 100 ml. cattle blood plasma. A composite curve from analyses of plasma from forty beef cattle including animals with vitamin A deficiency symptoms, animals on restricted levels of carotene intake and animals on normal rations including pasture feeding.

levels of carotene intake and others on normal rations including pasture in some cases.

Considerable variation is evident among the determinations made on the animals on the low-carotenoid ration. This is due in part to various stages of depletion of the carotene and

vitamin A reserves in these animals. Occasional animals had higher vitamin A values than were expected from the carotene content of the plasma and this was interpreted as indicating that the vitamin A reserve was still fairly adequate. However, when the animals continued with low blood carotene, deficiency symptoms developed with further reduction in blood plasma vitamin A.

Variation in the carotene and vitamin A content of the blood is also evident in animals on restricted levels and high levels of carotene intake. For example, two heifers received 30 and 60 µg. of carotene per kilogram of body weight daily during a period of more than 1 year. On the 30 µg. level the plasma carotene varied from 30 to 38 µg. per 100 ml. for heifer 173, and 48 to 58 µg. per 100 ml. for heifer 29. The corresponding range of vitamin A values on these two animals was 10 to 14 µg., and 19 to 21 µg. per 100 ml., respectively. Heifer 166 on the 60 µg. level of carotene varied in plasma carotene from 62 to 78 µg. and in vitamin A from 20 to 29 µg. per 100 ml., while heifer 98 received the same amount of carotene per unit of body weight and varied in plasma carotene from 84 to 96 µg. and in vitamin A from 23 to 31 µg. per 100 ml.

The reproductive performance and the carotene and vitamin A values of the blood plasma for the pregnant heifers shortly before calving are given in table 2. These plasma values are not included in table 1.

Heifers receiving 30 and 45 µg. of carotene per kilogram of body weight produced calves that were either blind and weak, or dead at birth. The calf from the heifer 78 on the 45 µg. level was stronger at birth than the calf from heifer 173 on the 30 µg. level and grew apparently normally but began to develop typical vitamin A deficiency convulsions at 21 days of age and died 23 days later. Both of the calves born to the animals on the 60 µg. level were apparently normal at birth. Heifer 98 had 96 µg. of carotene and 22 µg. vitamin A per 100 ml. plasma and her calf gained well and was still apparently normal at about 4 months of age. The other calf whose mother had less plasma carotene but with the same plasma vitamin A was

normal until about 1 month of age at which time he began to have occasional convulsions. Supplements of magnesium citrate, additional viosterol and carotene failed to correct the condition and the animal died about 2 months later.

Heifer 234 on the 120 µg. level of carotene, in contrast to the two heifers receiving only half as much carotene, aborted about the eighth month of pregnancy. A post mortem examination of this heifer was made 4 days after aborting and infantile ovaries and a cystic pituitary gland were observed. The plasma carotene and vitamin A of this animal were indicative of her higher level of carotene intake. The control animal,

TABLE 2

Reproductive performance, blood plasma carotene and vitamin A of individual pregnant heifers 1 to 7 days before calving when fed controlled levels of carotene

HEIFER NO.	CAROTENE INTAKE OF HEIFER	OBSERVED CONDITION OF CALF	BLOOD PLASMA	
			Carotene	Vitamin A
	µg./kg.		*µg./100 ml.*	*µg./100 ml.*
173	30	Weak; blind; convulsions	30	10
29	30	Died at birth	42	14
78	45	Blind; convulsions in 3 weeks	59	20
98	60	Very good	96	22
166	60	Good	78	22
234	120	Dead; premature birth	116 [2]	24 [2]
169	control[1]	Excellent	143	24

[1] Received 2 kg. U. S. No. 1 alfalfa hay daily.
[2] Plasma sample taken day after abortion.

heifer 169, with a slightly higher plasma carotene than the heifer receiving the 120 µg. level of carotene, but with the same concentration of plasma vitamin A, had a strong normal calf.

DISCUSSION

Analysis of cattle blood plasma for carotene and vitamin A by the procedure described indicates that long continued inadequate carotene intake and vitamin A deficiency in cattle can be determined by blood analysis. Preliminary findings reported by Mohler ('39), which include some of the data given in the present paper, indicate that the critical level of carotene

in the plasma is about 25 μg. per 100 ml. and for vitamin A in the same sample about 16 μg. per 100 ml. Animals having carotene and vitamin A values at these levels or above usually do not show the characteristic clinical symptoms of vitamin A deficiency.

These results with regard to the critical level of carotene are essentially in agreement with those of Moore ('39 b) who reports that plasma carotene at 0.2 μg. per milliliter or above was sufficient to prevent nyctalopia (nightblindness) and to maintain fair general health in Holstein and Ayrshire calves. In the same study, Moore noted that nyctalopia and papillary edema followed when the carotene values fell below about 0.13 μg. per milliliter. It is possible that the difference in the critical level of carotene in the plasma as found in this investigation and those of Moore may be partially accounted for by difference in the breed of cattle studied.

Plasma carotene has been shown to increase to higher levels with increasing carotene intake, but plasma vitamin A, as measured by the method described, tends to reach a less variable maximum concentration at a relatively early point. This may be due to a physiological balance between rate of carotene conversion into vitamin A and vitamin A utilization.

It is evident that much more carotene is needed and consequently higher plasma carotene and vitamin A levels are necessary for heifers to give birth to normal calves than to prevent the development of typical symptoms of vitamin A deficiency. The two heifers receiving 60 μg. of carotene per kilogram body weight from high grade alfalfa leaf meal were able to produce apparently normal calves at birth, but this level of carotene intake cannot be considered a safe level for practical recommendations since one of the calves later developed deficiency symptoms. However, these findings, although representing only a few animals, provide some initial information on the levels of plasma carotene and vitamin A that must be maintained in the pregnant animal in order to expect the birth of normal or deficient calves. Undoubtedly, the carotene intake of cattle which is sufficient to maintain approximately maximum vitamin A values in the plasma is more than suffi-

cient for the prevention of the usual symptoms of vitamin A deficiency and should be optimum for reproduction.

Converse and Meigs ('38) found that when timothy or clover hay was fed without pasture a daily intake of 80 to 100 mg. of carotene seemed sufficient to produce normal calves but that weak or dead calves were expected when the daily intake of carotene was 50 to 60 mg. These values are somewhat higher than those suggested by the limited number of cases studied in the present investigation. These results should not be considered as conflicting since the breed of animals used, source of carotene and other conditions of the experiment are not comparable.

SUMMARY

A method is described for the spectrophotometric determination of carotene and vitamin A in cattle blood plasma. The method is quite satisfactory for the determination of carotene but vitamin A values must be considered relative because of substances in the plasma extract which cause some interfering absorption.

Vitamin A deficiency in cattle or an inadequate intake of carotene resulting in depletion of the animal's reserve can be detected by blood carotene and vitamin A analysis. The carotene and vitamin A content of blood plasma are dependent on the carotene intake and previous storage of these factors. After cattle have been depleted of carotene reserves, the blood carotene level is dependent on the carotene intake although there is some individual variation. The vitamin A content of blood plasma is closely related to its carotene content. However, vitamin A values tend to reach a stable level and do not increase proportionally with increasing carotene intake.

Apparently normal calves were born in two cases to beef heifers that received 60 µg. of carotene from high quality dehydrated alfalfa leaf meal per kilogram of body weight previous to and throughout the gestation period. However, one of the calves gave signs of being deficient at about 1 month of age. Heifers receiving 30 and 45 µg. of carotene produced deficient calves although the cows themselves remained apparently normal.

Blood plasma carotene and vitamin A values are given for a wide range of carotene intake and for seven heifers which gave birth to deficient or normal calves during the experiment.

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EFFECT OF A.T.10 (DIHYDROTACHYSTEROL) ON RICKETS IN RATS PRODUCED BY HIGH-CALCIUM-LOW-PHOSPHORUS DIETS

ALFRED T. SHOHL AND SIDNEY FARBER

Departments of Pediatrics and Pathology, Harvard Medical School, and the Infants' and the Children's Hospital, Boston, Massachusetts

ONE FIGURE

A.T.10, an irradiation product of ergosterol, is known to have important effects upon calcium and phosphorus metabolism. This has been amply demonstrated not only by Holtz et al. ('34), its discoverers, who showed its ability to raise serum calcium concentration after removal of the parathyroids, but also by the studies of Albright and collaborators ('38, '39) on the metabolism of calcium and phosphorus in idiopathic hypoparathyroidism and in rickets. They have compared the effects of A.T.10 to those of vitamin D_2 and parathyroid extract. It is their thesis that vitamin D has two primary actions, to increase calcium absorption from the gastrointestinal tract and to increase phosphate excretion in the urine, whereas parathyroid hormone does not increase calcium absorption but does increase the phosphate in the urine markedly. According to these authors A.T.10 has the same two actions as vitamin D, but increases calcium absorption less and phosphate excretion more. In confirmation of this hypothesis it has been shown that A.T.10 will prevent or cure rickets in rats produced by a low-calcium-high-phosphorus diet in the same dosage that is without effect on the rickets produced by classical high-calcium-low-phosphorus diets (Shohl, Fan and Farber, '39). It has been reported by

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Harnapp ('35) that A.T.10 has 1/400–1/600 the antiricketic potency of vitamin D_2. This has been confirmed.[1]

The present study was made to test whether A.T.10 in any amount is incapable of curing rickets produced by high-calcium-low-phosphorus diets and if so, if the healing is identical with that produced by vitamin D. In other words, does it resemble parathyroid hormone, which intensifies the ricketic process or delays healing, or does it resemble vitamin D in its action?

Like both parathyroid hormone and vitamin D, A.T.10, when given in sufficient amounts, has toxic effects. In fact, this material has been standardized on the basis of "borderline toxic dose" (in this paper called "minimal toxic dose") which involves the loss of weight or death of mice. We have studied the quantitative relationship between the amounts of A.T.10 which produce therapeutic and toxic effects.

EXPERIMENTAL PROCEDURE

Young albino rats of 28 to 30 days of age and 48 to 53 gm. in weight [2] were divided at random into six groups of four each and placed upon the high-calcium-low phosphorus ricketogenic diet no. 2965 of Steenbock and Black (76% fresh ground whole yellow corn, 20% gluten flour, 3% $CaCO_3$, and 1% NaCl). The rats of group 1 served as rickets controls (negative controls); those of group 2 were fed protective doses of cod liver oil containing 7 International Units of vitamin D per rat per day (positive controls); those of groups 3, 4, 5, and 6 were given 5, 25, 125 and 375 µg. of A.T.10 [3] respectively, as shown in table 1. The rats which received the "normal" diet (Sherman diet B) were not litter mate controls; they were used only to show the order of magnitude of findings in normal animals. All supplements were given daily in 1 drop doses, put directly into the rat's mouth. All dilutions of A.T.10 were made with

[1] Unpublished data of Dr. O. W. Barlow of the Winthrop Chemical Co.

[2] Obtained from Sprague-Dawley Co., Madison, Wis.

[3] The A.T.10 used was obtained through the Division of Medical Research of the Winthrop Chemical Company.

sesame oil (which was the same oil used by the manufacturer in making the preparation) in such concentration that the daily dose could be given in a single drop. The animals were weighed weekly.

At the end of 20 days the animals were x-rayed and the next day were killed by ether anesthesia and bled from the femoral vessels. The pooled blood serum of each group was analyzed

TABLE 1

GROUP	DIET AND SUPPLEMENT[1]	BLOOD SERUM			BONES			
		Cal-cium	Phos-phate	Phos-phatase	Ash of fat free femur	Ash	X-ray[2]	Histology[2]
		mg./100 cc.	mg. P/ 100 cc.	Bodansky units	%	mg.		
	Normal	10.5	8.2	21.5	56.0	84	0	0
1	Rickets control	10.9	3.3	48.2	37.6	35	++++	++++
2	Cod liver oil, 8 units	12.4	6.2	34.3	48.0	48	0	0
3	A.T.10, 5 μg. (⅕ M.T.D.[3])	11.7	4.8	36.7	46.0	50	+++	++
4	A.T.10, 25 μg. (1 M.T.D.)	12.1	6.8	32.7	45.4	41	0	0
5	A.T.10, 125 μg. (5 M.T.D.)	12.6	8.7	27.3	53.0	56	0 h?	0 h
6	A.T.10, 375 μg. (15 M.T.D.)	16.0	6.8	24.8	47.5	48	0 h	0 h

[1] Normal diet was Sherman diet B. Groups 1–6 received diet no. 2965 of Steenbock and Black.

[2] The number of + signs indicates the severity of rickets. 0 means no rickets. h means hypercalcification at provisional zone of calcification.

[3] Minimal toxic dose.

for calcium, inorganic phosphate and phosphatase. The femurs of the right legs were dissected out and analyzed for water, fat, other organic matter and ash, by the methods previously described (Shohl et al., '39). The animals were then autopsied and the organs and bones preserved for histologic examination.

The experiment proceeded without known error. Daily observation disclosed little except that the animals which received the smallest doses of A.T.10 seemed more active than the rickets controls, and those which received the largest doses

were more torpid. The animals receiving the largest doses became progressively more emaciated. On the morning of the twenty-first day of the experiment one animal in this group was found moribund, and died before blood could be obtained for analysis.

<div align="center">RESULTS</div>

Weight. The weight curves given in figure 1 demonstrate that 125 and 375 µg. of A.T.10 per rat per day (5 and 25 minimal toxic doses) caused definite loss of weight. In group 5
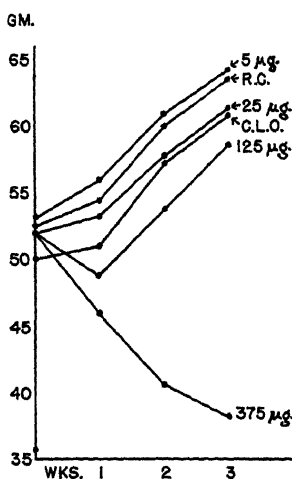
Fig. 1 Growth of rats fed high-calcium-low-phosphorus ricketogenic diet with supplements of A.T.10. R.C. = rickets controls. C.L.O. = cod liver oil supplement, 8 units daily.

this decrease was of only 1 week's duration, but in group 6 the weight loss continued, and one animal died. This must be regarded as definite evidence of toxicity. Further significance of the weight losses is discussed later.

Blood serum. The data are summarized in table 1. The rats of group 3, which received the smallest doses of A.T.10, showed higher calcium and phosphate values than the rickets controls. Each of groups nos. 4 to 6 received more A.T.10 than the one preceding it; correspondingly the serum calcium concentration of each group was higher than the one before it.

Only in group 6 was hypercalcemia marked. Similarly the serum phosphate concentration was greater with larger dosage, and approached normal values. The higher serum phosphate values were accompanied by lower phosphatase concentration.

Judged by these criteria alone the conclusion would be reached that the smallest doses of A.T.10 used (5 µg.) exerted a slight but definite antiricketic effect.

Bone analysis. Determination of the bone ash, which is probably the most sensitive method of demonstrating changes in calcium metabolism, shows the usual values for rickets controls and for the rats protected by cod liver oil (table 1). In all groups fed A.T.10 advanced mineralization took place. Even with the toxic doses the bones were not small in size, and they did not contain less ash, either absolutely or relatively than those of the rats which received cod liver oil. Judged by these analyses also it is concluded that 5 µg. of A.T.10 produce a definite antiricketic effect.

X-ray and histology. More weight is given to the interpretation of roentgenograms and histologic examination than to blood findings and bone ash (table 1).

In agreement with the roentgenograms, histologic examination of the tissues demonstrated that group 3 showed evidence of slight but definite antiricketic effect. Groups 2 and 4 showed marked healing. Groups 5 and 6 showed no rickets. Group 5 showed moderately dense, and group 6 very dense deposition of calcium mainly at the provisional zone of calcification, and to a lessened degree in the shaft. Thus all evidence is in agreement, except that in group 6 the bone ash is less than in group 5, both relatively and absolutely although hypercalcification is greater. This is interpreted as dissolution of bone salts from the shaft and their deposition at the provisional zone of calcification. The details of the histologic changes in this and related conditions will be described elsewhere. So far as could be determined the mechanism of healing of the ricketic lesion, following administration of A.T.10, was similar in all respects to that resulting from vitamin D.

DISCUSSION

From these data we conclude that, in the dosages used, A.T.10 protected rats from development of rickets produced by a high-calcium-low-phosphorus ricketogenic diet. Presumably the active agent was dihydrotachysterol. Unfortunately this cannot be stated definitely because the only product available is produced under German patents by secret processes abroad. There is no way of determining whether the effect of the preparation may be due to impurities such as other irradiation products of ergosterol or vitamin D itself. 'For this reason the term A.T.10 has been used rather than dihydrotachysterol.

It is well known that when rats receive food in inadequate amounts so that loss of weight occurs, healing of rickets takes place. Presumably this is due to the phosphorus supplied by the destruction of tissue. Under conditions in which weight losses occur it is therefore impossible to attribute the healing of rickets to the direct action of the drug studied. Therefore it is necessary to exclude from discussion the prevention of rickets in groups 5 and 6.

The factor of safety between the healing dose and the toxic dose of A.T.10 should be noted. It requires 0.0625 µg. of vitamin D to prevent rickets and 625.0 µg. will cause hypervitaminosis; with A.T.10, 25 µg. will afford protection, and 125 µg. will cause toxic symptoms. The toxic dose of vitamin D is approximately 10,000 times the therapeutic dose; with A.T.10 it is only about five times. Stated in other terms, A.T.10 is 1/400 as effective as vitamin D in curing of rickets and is five times as toxic.

The effect of A.T.10 on calcium and phosphorus metabolism is beyond question. In our previous study (Shohl et al., '39) with rickets produced by a low-calcium-high-phosphorus diet, healing resulted from an amount of A.T.10 approximately 1/80 the amount that was effective in this experiment. Thus the effect on calcium metabolism is demonstrable, under suitable conditions, with amounts which are much smaller than those which cause toxic manifestations. However, the highest

dosage used in the previous experiment (3 µg.) did not cause healing of rickets produced by a high-calcium-low-phosphorus diet. The smallest dose used in this experiment is approximately twice the largest dose used previously; the previous conclusions are therefore confirmed and extended.

In the previous experiment no increase in serum calcium concentration was found; the amounts of A.T.10 used in this experiment definitely raised serum calcium levels. Here again the property of increasing serum calcium concentration is found only when the dosage is increased to the point near which toxic symptoms appear.

Deposition of minerals may occur at the epiphysis when bone salts are being dissolved from the shaft and deposited at the provisional zone of calcification. This might take place without increase in the calcium absorption. Evidence of increased absorption is furnished when the balances of calcium and phosphorus are positive. However, equally good evidence of increased absorption is given in this case by the increase in the absolute amount of ash per femur. This can be present only if the balances are positive.

A.T.10 resembles vitamin D and differs from parathyroid hormone because it increases absorption of calcium, as shown by the fact that the bones of animals given A.T.10 increased in minerals both absolutely and relatively, compared to the rickets controls. If the principal effect of A.T.10 were to increase calcium absorption this would be most readily detectable when the calcium intake was high, but we previously showed that it was more effective in the cure of rickets when the phosphorus intake was high and the calcium low. Therefore, A.T.10 exerts its effect principally on the excretion of phosphate in the urine; and to a lesser extent on the absorption of calcium. This is in agreement with the hypothesis presented by Albright et al. ('38, '39).

Histologic examination, although it cannot differentiate between this type of healing and that produced by vitamin D, shows clearly that in this case, as well as with low-calcium diets, the prevention of rickets with A.T.10 can be accom-

plished without toxic manifestations. This indicates further that whatever may be the mechanism of deposition of minerals in uncalcified cartilage, in these and similar conditions it is primarily the calcium and phosphorus metabolism that determines whether rickets is present or not.

SUMMARY

A.T.10 in amounts which approximate toxic dosage, is effective in prevention of experimental rickets in rats fed a high-calcium-low-phosphorus ricketogenic diet.

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THE CALCIUM CONTENT OF THE NORMAL GROWING BODY AT A GIVEN AGE

K. E. BRIWA AND H. C. SHERMAN

Department of Chemistry, Columbia University, New York

THE PROBLEM

An outstanding feature of the chemistry of nutrition is the fact that the body, in its normal growth and development, must increase not only the amount but also the percentage of calcium which it contains. The purpose of the present work is to ascertain whether this normal increase in the percentage of calcium in the growing body is inherently related to age, or is significantly influenced by differences in growth rate within the normal zone.

Liebig taught the view that the individual plant or animal grows only at the rate or to the extent consistent with the specific chemical composition of its kind. From this it would follow that the rate of growth would be set by any substance or element essential to the body composition which is present in minimal proportion relative to need for growth at a normal rate. Hence this idea has often been called Liebig's "Law of the Minimum." Now after nearly a century, we can see that some aspects of growth and development do "obey" this so-called law while other aspects do not. Thus among the many published investigations of the effects of shortages of specific amino acids we find no report of animals growing-up with, for instance, lysine-poor bodies; while there is no lack of evidence that shortage of calcium in the food may result in a calcium-poor body.

Aron and Sebauer ('08) published observations upon dogs of essentially the same age and size, one of which had grown

up on normal, the other on calcium-poor food. The fresh bones of the normal and calcium-poor animals of the same size weighed about the same; but upon drying, those of the calcium-poor dog were found to contain more water and less calcium than those of dogs receiving food of normal calcium content.

Osborne and Mendel ('18) showed that some growing animals on a calcium-poor diet were promptly checked in growth while others grew some time before showing any outwardly visible effects of calcium deficiency.

Sherman and Hawley ('22) in work with children found that those with the higher calcium intake stored more and thus must have had a higher calcium content at a given age.

Moulton ('23) studied the composition of the ox and man from early embryonic stage to maturity. The water content of these mammals decreased very rapidly from conception to birth, and then less rapidly until in each case a stable composition was reached. This condition, of a stabilized or plateaued chemical composition of body, Moulton called "chemical maturity"; this term thus referring to the percentage and not to the amount of the given chemical constituent in the body.

Sherman and MacLeod ('25) found the body calcium of normal rats to increase from about 0.25% at birth to about 1.25% at maturity. Even in this long-controlled, laboratory-bred colony there is (as in a human population) considerable individual variability in the rate of growth.

The problem of the investigation reported in this paper was therefore to find whether in the normal animal it is primarily the age or the individual rate of growth which determines the percentage of calcium in the body; or whether progress toward "chemical maturity" in Moulton's sense is (in the case of calcium) determined essentially by age or by growth. This problem has been given increased importance by the reference of Shohl ('39) to the change in the body's composition as correlated with its increase in size, though the context does not indicate that discrimination between age and size was definitely intended.

EXPERIMENTAL

The experimental animals were albino rats of Osborne-Mendel stock which during its period of about fifty generations in the Columbia laboratory has been bred from average members of each successive generation and so has not increased so much in individual size as have the rats of the many colonies in which the tendency is to select animals of superior size for breeding.

In the present work, all the members of a given litter were killed for analysis at the age of 28 days, which in terms of human analogy may be considered as the end of infancy. This was the age at which largest numbers of animals were available for our analyses, and it is well within the period of rapid growth in which a distinction between age and size should have its greatest significance. Each sex was considered as constituting a separate series; and as animals from families on three different diets were treated separately, there were in all six series of analyses each of animals homogeneous except for individual differences in growth and resulting size at a given age.

We wish to make grateful acknowledgment to Dr. H. L. Campbell of this laboratory, under whose direction the animals were reared.

The three dietaries or family nutritional backgrounds were: (1) diet 13, consisting of two-thirds ground whole wheat and one-third dried whole milk, and containing 0.35% of calcium in the dry food mixture; (2) diet 132, differing from diet 13 in that enough calcium carbonate was added to bring the calcium content of the dry food to 0.49%; and (3) diet 133 in which enough more calcium carbonate was added to bring the calcium to 0.64% of the dry weight of the food. These food-mixtures, each of which contained from 0.49 to 0.50% of phosphorus and from 16.1 to 16.2% of protein, were fed with sodium chloride and distilled water.

The results of the analyses of the animals of each of the six series (each sex from each of the three family diets) were studied statistically, for possible influence of body size at the given age, in two ways: first, by averaging in three groups (table 1) the "large", "medium", and "small" members of each litter, with about half of the cases assigned to the medium

TABLE 1

Calcium contents of 28-day-old rats grouped according to size as the small, medium, and large members of their respective litters

SEX AND NUTRITIONAL BACKGROUND		SIZE	NO. CASES	NET BODY WEIGHT	BODY CALCIUM	
				gm.	gm.	% and P.E.[1]
M.	Diet 13	Small	16	40.4	0.299	0.74 ± 0.005
		Medium	31	46.0	0.336	0.73 ± 0.004
		Large	16	55.5	0.396	0.71 ± 0.003
M.	Diet 132	Small	16	37.1	0.305	0.82 ± 0.008
		Medium	32	43.9	0.350	0.80 ± 0.004
		Large	16	53.2	0.422	0.79 ± 0.004
M.	Diet 133	Small	18	37.5	0.326	0.87 ± 0.005
		Medium	36	47.3	0.406	0.86 ± 0.005
		Large	17	54.7	0.446	0.82 ± 0.006
F.	Diet 13	Small	18	38.2	0.295	0.77 ± 0.004
		Medium	33	43.5	0.326	0.75 ± 0.004
		Large	19	49.9	0.370	0.74 ± 0.005
F.	Diet 132	Small	20	33.5	0.282	0.84 ± 0.006
		Medium	34	41.4	0.338	0.82 ± 0.004
		Large	18	48.6	0.400	0.82 ± 0.004
F.	Diet 133	Small	20	35.1	0.321	0.91 ± 0.006
		Medium	40	44.4	0.397	0.89 ± 0.005
		Large	20	52.2	0.444	0.85 ± 0.006

[1] P.E. = probable error of the mean, as fully explained by Chaddock ('25).

group; and second, by averaging in two groups consisting respectively of those above and those below the mean weight of their litter and designated (in table 2) as "heavier" and "lighter". The animals were prepared and cremated, and their calcium contents determined by the McCrudden method, with the precautions described by Sherman and MacLeod ('25).

DISCUSSION

For economy of space the coefficients of variation are not tabulated, since they are all of the order of 3 to 5 with no consistent indication that either the smaller or the larger animals were more variable than the average of their series. Hence, also, variability was satisfactorily low in all groups and the conclusion to be drawn as to the relative influence of age and size is strengthened. As the direct relation of body calcium to age was established by the work of Sherman and MacLeod ('25), the problem here becomes whether or not the percentage at a given age is measurably influenced by differences (within the normal range) in the rate at which the individual has grown as shown in the weight of the body at a given age.

All six of the comparisons summarized in table 1 show slightly higher percentages of calcium in the "small", and five of the six show slightly lower percentages in the "large", than in the "medium" or average of their respective age, sex, and nutritional background. All these differences are, however, so small that none of them considered independently would be regarded as statistically significant.

For further statistical scrutiny, the animals of each of the six series were divided again according to size but into only two groups: the "heavier" and "lighter" halves. The numerical findings as summarized in table 2 show higher mean percentages of calcium in the lighter animals in all six of the comparisons. The differences, however, appear here as in the previous mode of interpretation to be insignificant compared with such differences as have been found due to age or to level of calcium intake. If the ratio of each difference to its probable error be taken alone and given the conventional interpretation, half of them would on the face of the figures appear "significant" and half "insignificant"; but in the light of all the evidence, and particularly of the fact that all the differences are near the limit of accuracy of the best experimental and analytical methods, it does not seem best in this case to say that some of the differences are significant and others are

not. Neither a separate comparison according to sex nor a separate comparison according to nutritional background shows any consistent trend of difference. There is probably a very slight inverse relationship between the size of the body and its percentage of calcium at the given age here studied; but this, if it exists, is so small a factor as to be only doubtfully measurable, and practically insignificant as compared with the age factor.

TABLE 2

Calcium contents of 28-day-old rats grouped according to size as the ''lighter'' and ''heavier'' halves of the individuals of their respective series

SEX AND NUTRITIONAL BACKGROUND		SIZE	NO. CASES	NET BODY WEIGHT	BODY CALCIUM	
				gm.	*gm.*	% and P.E.[1]
M.	Diet 13	Lighter	31	42.2	0.310	0.74 ± 0.004
		Heavier	32	51.6	0.372	0.72 ± 0.003
M.	Diet 132	Lighter	32	39.6	0.321	0.81 ± 0.005
		Heavier	32	49.5	0.393	0.79 ± 0.004
M.	Diet 133	Lighter	36	41.3	0.360	0.87 ± 0.004
		Heavier	35	52.0	0.431	0.83 ± 0.005
F.	Diet 13	Lighter	36	40.0	0.306	0.76 ± 0.004
		Heavier	34	47.9	0.356	0.74 ± 0.004
F.	Diet 132	Lighter	37	36.5	0.303	0.83 ± 0.004
		Heavier	35	45.8	0.376	0.82 ± 0.003
F.	Diet 133	Lighter	38	38.6	0.352	0.91 ± 0.004
		Heavier	42	48.9	0.423	0.87 ± 0.004

[1] P.E. = the probable error of the mean.

Hence whichever the mode of comparison and interpretation of the data, it appears that the percentage of body calcium in the normal growing animals of the same sex and nutritional background, was essentially determined by their age, the smaller animals containing only a very slightly higher percentage of calcium than the larger animals of the same age and sex.

As all of the foregoing discussion is in terms of percentages of body calcium, it obviously follows that the amount of calcium in the body was larger in the larger rats and smaller in the smaller rats, almost in proportion to their body weight.

SUMMARY AND CONCLUSION

Normal animals at a given age were compared (males and females separately) as to whether the percentage of body calcium differed materially with the body weight of the animal; i.e., with the rate at which it had grown. Such comparisons were made independently with animals from three different diets representing successive levels of liberality of calcium intake. Thus there were six comparisons, each embracing from sixty-four to eighty animals.

In none of these six comparisons did the larger animals show a higher percentage of body calcium than the smaller animals of the same age, as would be the case if the higher body weight meant greater maturity of skeletal development at the age studied (28 days in the rat, or "end of infancy").

Obviously the amount (total weight) of calcium in the body tends to vary with its size. The comparisons which follow are therefore made on the basis of percentage of body calcium.

In all of the six comparisons, the smaller animals show a slightly higher, and the larger animals a slightly lower, percentage of body calcium than the general average of their group. The differences, however, were only such as might be regarded as being near the limit of accuracy of the method of experiment and analysis, and so on the border line of possible statistical significance.

Thus the rate of growth as expressed by the body weight at a given age, is in itself not a disturbing factor in the rate of calcification involved in normal development. This finding is of special significance in the interpretation of certain investigations upon the influence of food.

These analyses of well over 400 experimental animals establish the fact that among normal growing individuals of a given sex, having the same hereditary and nutritional background, age is the predominant determining factor in the increasing percentage of calcium in the body.

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A QUANTITATIVE STUDY OF VITAMINS IN THE RUMEN CONTENT OF SHEEP AND COWS FED VITAMIN-LOW DIETS

III. THIAMIN

L. W. McELROY AND H. GOSS

*Division of Animal Husbandry, College of Agriculture,
University of California, Davis*

ONE FIGURE

Early investigations (Theiler et al., '15; Bechdel et al., '26, '27, '28) dealing with the vitamin B requirements of ruminants were handicapped by the existing lack of knowledge regarding the complex nature of the B vitamins and by the lack of well-defined methods of assay. Reinvestigation of the problem in the light of more recent knowledge is therefore timely. It was previously reported (McElroy and Goss, '39) that thiamin was synthesized in the rumen of sheep. The animals were fed a ration deficient in thiamin and their rumen and reticulum contents were found to contain more than 5 µg. of thiamin per gram. Additional assays have been made to determine more accurately the thiamin potency of this sheep rumen and reticulum material and also to determine if a similar synthesis of thiamin occurs in the rumen of the cow.

EXPERIMENTAL

The ruminant ration and the method used to obtain samples of rumen contents from the experimental animals were the same as described in previous papers (McElroy and Goss, '40 a, '40 b).

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Thiamin assays of sheep rumen contents. Thiamin assays were conducted by the method of Jukes and Heitman ('40), in which the degree of postponement of fatal polyneuritic symptoms afforded by the test material is compared with that afforded by graded levels of synthetic thiamin chloride hydrochloride [1] added to chick diet 152, deficient in thiamin.

In the first thiamin assay the dried sheep rumen and reticulum contents were fed at a level of 20%. Shortly after the
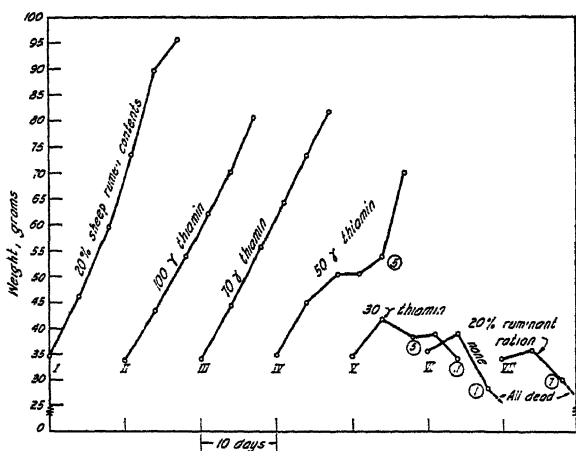
Fig. 1   Growth curves of chicks in the first thiamin assay of ruminant ration and of dried rumen and reticulum contents. Twelve chicks were included in each group. The figures in circles under the curves represent the cumulative number of deaths from polyneuritis. The last part of the growth curve for groups VI and VII is shown as a broken line since no weights were taken during this interval. Supplements added to basal diet 152 are shown above the curves. The amounts of thiamin indicated were added per 100 gm. of diet.

beginning of the trial it became evident that the thiamin potency of the test material had been underestimated and the assay was stopped on the seventeenth day since it had become obvious that the rumen material contained over 5 µg. of thiamin per gram and it would be necessary to feed it at a lower level in order to obtain an assay. The growth made by the different groups up to the seventeenth day is shown in figure 1. When the trial was stopped the chicks in groups I

[1] Merck and Company.

and II were changed to the basal diet and further evidence for the high thiamin content of the sheep rumen and reticulum material was supplied by the fact that the average postponement of fatal polyneuritis in the group fed sheep rumen and reticulum contents was 13.1 days as compared to 8.3 days for the group which had previously received 100 µg. of thiamin per 100 gm. of diet. Jukes and Heitman ('40) have estimated that diet 152 contains less than 0.4 µg. of thiamin per gram. The results for groups VI and VII indicate that the ruminant ration did not contain appreciably any more thiamin than diet 152 since supplementing this diet with 20% ruminant ration failed to delay the onset of fatal polyneuritis.

TABLE 1

Thiamin assay of ruminant ration and sheep rumen contents

GROUP NO.	AMOUNT OF SUPPLEMENT ADDED TO BASAL DIET 152	NUMBER OF CHICKS AT BEGINNING OF TRIAL	GROUP POLYNEURITIC MORTALITY INDEX [1]
I	0	12	19.0
II	5% ruminant ration	12	18.8
III	5% sheep rumen and reticulum contents	12	14.9
IV	50 µg. thiamin per 100 gm.	12	9.4
V	70 µg. thiamin per 100 gm.	12	5.6

[1] The "polyneuritic mortality index" was calculated for each chick by subtracting the number of days it survived from the number of days (28) in the assay period. Complete protection was therefore indicated by an index value of zero.

The results of the second thiamin assay of sheep rumen and reticulum contents in which the test material was fed at a level of 5% are summarized in table 1. From the data in table 1 and the response curve of Jukes and Heitman ('40) it was estimated that the sheep rumen and reticulum contents contained approximately 7 µg. of thiamin per gram.

This represents a seventeenfold increase in thiamin potency for the rumen and reticulum contents over the ruminant ration and reasons have been presented in a previous communication (McElroy and Goss, '40 b) for considering increases of this magnitude as evidence of vitamin production,

presumably by microbial growth, rather than of a simple concentration of vitamin already present in the ration.

<center>EXPERIMENTS WITH COWS</center>

In preliminary work a pure bred Jersey cow with a permanent fistula into the rumen was used. Considerable difficulty was encountered in getting this cow to accept the experimental feed without a supplement of a small amount of alfalfa hay and when, after 5 weeks, hay was omitted entirely from the ration, severe anorexia developed after 8 days and the cow refused to eat any of the experimental feed. At this time a sample of rumen contents was removed for drying and subsequent biological assay and the cow was turned out to pasture to recover. However, neither her appetite nor rumen activity was restored and 4 days later she died with symptoms suggestive of thiamin deficiency paralysis. A post mortem examination failed to reveal any acute condition other than putrefaction of the rumen contents. No thiamin could be detected by biological assay with chicks in the sample of rumen contents removed before the cow was turned out to pasture. These results suggested the possibility that thiamin deficiency may have been the primary cause of complete failure of appetite and subsequent death of this animal.

Since, in the experiments with sheep described above, no cases of anorexia developed and subsequent assays indicated the synthesis of an appreciable amount of thiamin in the intact rumen of this species, results of the opposite nature with the bovine had not been anticipated. One possible explanation of the difference appeared to be that the presence of a fistula into the rumen of the cow might have had an inhibitory action on the growth of rumen organisms capable of synthesizing thiamin, and this combined with loss of rumen fluid through leakage might have contributed to a severe thiamin deficiency. Therefore, in order to test the possibility that a fistulated animal might not be a satisfactory subject for this type of experiment, one normal or non-fistulated cow,

no. 706, and one fistulated cow, no. 557, were used in subsequent studies.

Both of these cows accepted the experimental ration much more readily than did the cow used in the preliminary trial. Nevertheless anorexia developed at intervals during the course of the experiment. The normal cow, no. 706, went off feed twice during the 3 months she was fed the vitamin deficient ration, but both times regained her appetite after starving for 18 to 36 hours. The fistulated cow, no. 557, refused to eat for periods of 24 to 56 hours six times during the 5 months of experimental regime.

In view of the previous experience which suggested that the cause of anorexia might be thiamin deficiency, intravenous thiamin injections were tried on the three occasions when no. 557 refused to eat for more than 36 hours. Successively larger doses of 50, 100 and 500 mg. of thiamin were used. The results were indefinite. When the 50 mg. injection was given the cow refused to eat 1 hour after the injection, but accepted the feed after 6 hours. It was thought that perhaps a larger dose would produce a more rapid and marked desire to eat, but when the dose was increased to 100 and 500 mg. on succeeding occasions the cow failed to eat until 8 and 14 hours respectively after the injection was made. The possibility that the cow would have accepted the experimental feed just as soon without having been injected with thiamin cannot be eliminated. Nevertheless the following observations may be significant:

1. The rumen was either quiescent or very sluggish before the injections were made but became active within 1 to 3 hours after injection.

2. In the case of the first and second injections the cow was noted to lick the empty feed trough and exhibit signs of hunger after 3 to 4 hours, even though she still refused to eat when feed was placed before her.

3. Before injection the muzzle was dry and there was a thick discharge from the nostrils. The muzzle always became

moist within a few hours after the injection and the nasal discharge disappeared within 24 to 72 hours.

Samples of rumen contents were taken from this cow only when her appetite was good and, as will be shown later, no thiamin was detected in these samples.

With regard to the non-fistulated cow used as a control, we were not successful in obtaining a satisfactory sample of her rumen contents for biological assay. After this cow had been on the experimental diet for 88 days she was slaughtered for the purpose of obtaining her rumen contents but unfortunately the bulk of the rumen material was lost during the slaughtering operation. Only a small amount of material retained in the folds of the rumen was saved and as this contained a considerable proportion of sand and small bits of gravel it was by no means a representative sample of the organic matter present in the rumen of the animal during the experimental period.

Thiamin assays of cow ration, rumen contents, and skim milk from cow no. 557. As mentioned in an earlier paper (McElroy and Goss, '40 a) fistulated cow no. 557 calved during the course of the experiment and consequently samples of both her rumen contents and milk were available for thiamin assay. In preparation for thiamin assay the milk was first skimmed and then concentrated 4.7:1 under reduced pressure at a maximum temperature of 38°C. This concentrate was subsequently mixed with a known weight of dry basal diet 152 and dried to a constant weight in a current of air at 40°C. after which sufficient diet 152 was added to lower the concentration of dried skim milk to 15% or 23% as desired. A sample of commercial skim milk powder prepared by the drum process was assayed at the same time. The results of the assay are summarized in table 2.

Table 2 shows that no thiamin was detected in the ruminant ration by biological assay with chicks. This is in agreement with results shown in table 1 and figure 1. In contrast to the earlier results which showed that dried rumen and reticulum contents from sheep fed the deficient ration contained approxi-

mately 7 µg of thiamin per gram, the results shown in table 2 indicate that the dried rumen contents of cow no. 557 contained no more thiamin than basal diet 152, or less than 0.4 µg. per gram. Later assays in which dried rumen material from cow no. 557 was fed at levels of 20% and 30% substantiated this conclusion.

Despite the fact that no thiamin was detected in the rumen contents of cow no. 557 it may be seen from table 2 that her milk contained an appreciable amount of the vitamin. From the polyneuritic mortality index values for groups VI and

TABLE 2

Thiamin assay of ruminant ration, rumen contents and dried skim milk

GROUP NO.	SUPPLEMENT ADDED TO BASAL DIET 152	NUMBER OF CHICKS AT BEGINNING OF ASSAY	POLYNEURITIC MORTALITY INDEX
I	0	10	17.7
II	14% ruminant ration	10	18.5
III	7% rumen contents from no. 557 dried at pH 4.5 to 5.0	10	18.2
IV	14% rumen contents from no. 557 dried at pH 4.5 to 5.0	10	16.8
V	15% dried skim milk from no. 557	10	15.5
VI	23% dried skim milk from no. 557	10	7.0
VII	20% University Farm skim milk powder	10	6.4
VIII	50 µg. thiamin per 100 gm.	10	7.4
IX	80 µg. thiamin per 100 gm.	10	4.4

VIII the thiamin potency of dried skim milk from the experimental cow was estimated to be between 2 and 2.5 µg. per gram. From the index value of 6.4 for group VII it was estimated that the thiamin content of this sample of commercial skim milk powder was about 3 µg. per gram.

The fact that no thiamin was detected in the rumen contents while an appreciable quantity of the vitamin was secreted in the milk appeared explainable in either of two ways. First, thiamin synthesized in the rumen may have been destroyed during processing of the rumen contents or second, thiamin synthesis may have occurred in sections of the digestive tract posterior to the rumen. As described in a previous com-

munication (McElroy and Goss, '40 a) the method used in preparing cow rumen samples differed from that employed for sheep rumen samples in that the cow rumen contents were dried in an oven before a current of air at 40° to 50°C. after the addition of ethyl alcohol to make the concentration of alcohol 30% and the addition of HCl to make the pH 4.5 to 5.0, while the sheep material was dried at room temperature (not exceeding 30°C.) after the addition of enough alcohol to make the concentration about 50%. No acid was added to the sheep rumen and reticulum contents. Sheep rumen and reticulum contents treated in this way yielded a product which assayed 7 µg. of thiamin per gram, but samples of the rumen contents from cow no. 557 treated by the method used for the material from the sheep showed no more thiamin activity than

TABLE 3

Thiamin assay of dried cow rumen contents from cow no. 706

GROUP NO.	SUPPLEMENT ADDED TO BASAL DIET 152	NUMBER OF CHICKS AT BEGINNING OF ASSAY	POLYNEURITIC MORTALITY INDEX
I	0	12	18.1
II	15% rumen contents dried at pH 4.5	12	13.8
III	50 µg. thiamin per 100 gm.	12	11.2

did samples prepared in the regular way. It would therefore appear that destruction of thiamin during drying cannot be offered as a major factor in explaining the low level of the vitamin in the rumen contents of fistulated cow no. 557.

As has been previously stated, the sample of rumen contents obtained from non-fistulated cow no. 706 was not representative of the organic matter present in the rumen but as shown in table 3 the addition of 15% of this material to diet 152 served to delay the onset of fatal polyneuritis in chicks. From table 3 and the response curve of Jukes and Heitman ('40) it was estimated that this sample of rumen contents from cow no. 706 contained approximately 2.5 µg. of thiamin per gram. This figure is undoubtedly considerably lower than that which would have been found had it been possible to assay a

sample from this cow of rumen material which was free of foreign inorganic matter. Although more comprehensive study would be required to prove the point, the response of fistulated cow no. 557 to thiamin injections and the results of the thiamin assays of rumen contents from the fistulated and non-fistulated animals suggest that the presence of an artificial opening into the rumen may alter the habitat in such a way as to make it unfavorable for the growth of rumen organisms capable of synthesizing thiamin.

DISCUSSION

Since the dried rumen and reticulum contents of sheep fed a thiamin deficient ration were found to be a good source of thiamin it is concluded that under the conditions of this investigation microorganisms present in the rumen of the sheep were able to synthesize the vitamin. In view of these results it seems reasonable to assume that the development of a state of thiamin deficiency under conditions of practical sheep husbandry is very improbable, even though it is not established that thiamin synthesis will take place in the rumen of sheep regardless of the composition of the deficient diet fed.

Scheunert and Schieblich ('23) demonstrated that B. vulgatus, an obligate intestinal organism of herbivors, is capable of synthesizing vitamin B_1. Theiler, Green and Viljoen ('15) and Bechdel and co-workers ('26, '27, '28) have conducted pioneer investigations in feeding vitamin B complex deficient rations to cattle. In the main the results of our cow experiments are in agreement with those of these early investigators in indicating that thiamin may be synthesized in the rumen of the bovine and is therefore not a dietary essential for this species.

In their work with a rumen fistula cow, Bechdel, Honeywell, Dutcher and Knutsen ('28) found that after the preparation of the fistula "the heifer continued to thrive (on the ration deficient in vitamin B) and gave every evidence of functioning normally as she had done on the experimental ration

prior to the operation.'' While this offers no support for our suggestion that the presence of an artificial opening into the rumen may result in a decreased thiamin synthesis it does not eliminate the possibility, particularly since their ultimate vitamin assay of the rumen contents was conducted, not with the dried rumen contents, but with a concentrated alcoholic extract of rumen material which had been incubated for 5 days ''in order that the bacterial flora might multiply and produce, if possible, the maximum quantity of vitamin B.'' One gram of their final extract represented 25.4 gm. of the original fresh fermented rumen material and the extract was fed at a level of 50% of the diet, so that the concentration of the vitamin in the original rumen contents need not necessarily have been high in order to produce the growth response which they obtained with rats.

SUMMARY

1. Four sheep were fed a ration containing less than 0.4 µg. of thiamin per gram. The dried rumen and reticulum contents of the sheep were found to contain approximately 7 µg. of thiamin per gram.

2. No thiamin was detected in the rumen contents of two fistulated cows fed the same deficient ration, but the milk of one of these cows contained between 2 and 2.5 µg. per gram on a dry basis.

3. Thiamin was detected in the rumen contents of a non-fistulated cow fed the deficient ration.

4. These results confirm and extend the work of earlier investigators in indicating that thiamin is not a dietary essential for ruminants.

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RIBOFLAVIN CONTENTS OF SOME TYPICAL FRUITS[1]

CAROLINE SHERMAN LANFORD, BEATRICE FINKELSTEIN AND
H. C. SHERMAN

Department of Chemistry, Columbia University, New York City

The quantitative distribution of riboflavin in natural food materials is of interest from two points of view. The fact that riboflavin functions importantly in the tissue respiratory and perhaps other processes of both plant and animal nutrition gives scientific significance to a knowledge, as broad and as precise as is practicable, of the relative concentrations of this substance in different plant and animal tissues. The relative richness of different foods in riboflavin is also brought closer to the scientific interests and professional responsibilities of workers in nutrition by the present-day knowledge that shortage of riboflavin in the dietary is a not infrequent human nutritional deficiency disease (Sebrell and Butler, '39; Oden, Oden and Sebrell, '39; Sydenstricker, Geeslin, Templeton and Weaver, '39; Kruse, Sydenstricker, Sebrell and Cleckley, '40; Sydenstricker, Sebrell, Cleckley and Kruse, '40).

Two citrus and two pome fruits and one species of each of two other families, including the tomato as being botanically a fruit though often called a vegetable, are reported upon in this paper.

MATERIALS AND METHODS

In the case of apple and of pear, the "edible portion" was represented by a section of the fresh flesh of the fruit with its proportionate amount of skin, but without seeds. Banana was fed as sections of the fresh fruit with skin removed. Orange

[1] The aid of a grant from the Florida Citrus Commission is gratefully acknowledged.

was fed as fresh fruit with skin and seeds but not septum removed; grapefruit, after removal of skin, seeds, and septum. Tomato was fed as the mixed juice and pulp of canned tomato after passing through a coarse sieve to remove skins and seeds only. Canned grapefruit juice was also tested.

The determinations of riboflavin here reported were all made by the feeding method, using young rats essentially as described by Bourquin and Sherman ('31) with the added precaution of harnessing for complete prevention of coprophagy

TABLE 1

Riboflavin contents of certain fruits

FRUIT	NUMBER OF TEST ANIMALS	MICROGRAMS OF RIBOFLAVIN FOUND:	
		per 100 gm. Mean ± P.E.[1]	per 100 Calories Mean ± P.E.[1]
Apple	14	4.3 ± 1.2	7.7 ± 2.1
Banana	12	47.7 ± 4.6	48.7 ± 4.7
Grapefruit	26	20.0 ± 2.1	45.5 ± 4.8
Grapefruit juice (canned)	12	11.8 ± 1.4	(see text)
Orange	17	27.8 ± 2.0	55.6 ± 4.0
Pear	14	19.8 ± 2.8	28.3 ± 4.0
Tomato	13	37.3 ± 3.3	162.4 ± 14.3

[1] P.E. = the probable error of the mean.

as described by Page ('32), and with all tests controlled by strictly parallel (side-by-side) feedings with pure riboflavin. The 4-week test period was used.

FINDINGS AND CONCLUSIONS

The findings are summarized in table 1, as micrograms of riboflavin both per 100 gm. and per 100 Calories of the fresh fruit (the latter, however, omitted in the case of canned grapefruit juice because of differing customs as to presence or absence of added sugar).

Compared either per 100 gm. or per 100 Calories of fresh edible material, the citrus fruits (orange and grapefruit), banana, and tomato appear richer in riboflavin than the pome fruits as here represented by apple and pear. Considered with reference to the amounts presumably involved in average

human nutrition (Stiebeling and Phipard, '39) each 100-Calorie portion of citrus fruit (grapefruit or orange) or of banana contributes its full quota of riboflavin to the dietary, while the 100-Calorie portion of tomato contributes more, and that of pome fruit (apple or pear) contributes less.

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EFFECTS OF PARATHYROID DEFICIENCY AND CALCIUM AND PHOSPHORUS OF THE DIET ON PREGNANT RATS [1]

M. BODANSKY AND VIRGINIA B. DUFF

The John Sealy Memorial Research Laboratory and the
University of Texas Medical School, Galveston

This paper is devoted to a comparison of the course of pregnancy in normal and parathyroid-deficient rats and includes data on the composition of the blood at term. These observations are related to the endocrine deficiency and to the calcium and phosphorus content of the diet.

The choice of the albino rat (inbred Wistar strain) for these experiments has, aside from the advantages generally recognized by students of nutrition, the added and distinct advantage that in this animal complete extirpation of the parathyroids may be accomplished with a high degree of probability. The rat possesses, as a rule, two parathyroids, situated at the upper poles of the thyroid gland on the antero-lateral surface of each lobe. They are well encapsulated and are removable by micro-dissection without serious damage to the thyroid or other contiguous structures. Though certain of the earlier investigators assumed the existence of accessory parathyroid tissue, there now seems to be general agreement with the conclusion of Hoskins and Chandler ('25) that the occurrence of accessories is so infrequent as to be negligible in properly controlled experiments. Aberrant parathyroid tissue, when present, is usually located cephalad to the thyroid and dorsal to the cartilage of the larynx.

A possible limitation to the use of the rat in studies involving maternal and fetal mineral metabolism is the cartilaginous nature of the rat skeleton at birth, as compared to that of the human infant at birth. The per cent of ash of the rat pup is only about half that of the newborn infant; the calcium content per unit of body weight is only about a third, while the phosphorus content is in the neighborhood of 60%. As Cox and Imboden ('36 b) have shown, it requires approximately 21 days of nursing to raise the total ash, calcium and phosphorus of the rat pup to levels comparable to those of the human infant at birth.

These comparisons do not signify, however, that gestation per se involves less strain on the mineral stores of mother rats than obtains in human mothers, for it is only necessary to take into account that an average litter, weighing 50 gm., may be equivalent to one-fifth or even one-fourth of the maternal weight. Even though the per cent of calcium in a fetus is relatively low, the total calcium of a litter usually amounts to 5%, or more, of the total calcium of the maternal organism. In comparison, the average human infant, at birth, contains 24 to 30 gm. of calcium, which is in the neighborhood of 1.5 to 2.5% of the calcium estimated to exist in the average woman of child-bearing age.

The results of parathyroid deficiency are so significantly affected by the mineral composition of the diet that it seemed worth while to conduct our studies at different levels of calcium and phosphorus intake. The basal diet and the various experimental diets consisted of purified foodstuffs and were identical in composition with those employed by Cox and Imboden ('36 a) in their investigation of the role of calcium and phosphorus as determinants of reproductive success in normal rats. The composition of the basal diet is given in table 1.

Following the procedure given by Cox and Imboden, predetermined quantities of dicalcium phosphate and either monobasic ammonium phosphate or calcium acetate were added to the basal diet, thereby bringing the calcium and phosphorus

to the desired levels. Eight diets representing a comparatively wide range of variation of the calcium and phosphorus intake (see table 2) were employed in our studies.

Cox and Imboden ('36 a) determined reproductive success on the basis of the following criteria: (1) average weight of

TABLE 1

Composition of basal diet

	gm.
Casein (Labco, acid washed vitamin free)[1]	20.0
Lard (Swift's Silverleaf)	9.0
Yeast concentrate [2]	4.0
Wheat germ oil	1.6
Carotene solution in oil 3: 1000	0.3
Salts — Ca and P free [3]	3.1
Rice cellulose [4]	5.9
Dextrinized starch, after adding calcium-phosphorus mixtures, to make	100.0

[1] Purchased from Casein Company of America.

[2] We are indebted to the Mead Johnson Company, Evansville, Indiana, for the large quantities of yeast concentrate used in this and subsequent studies.

[3] Composition: NaCl 24.50, $MgSO_4$ 9.14, $KHCO_3$ 59.20, KCl 3.84, Fe citrate 2.94, $CuSo_4$ 0.32, $MnSO_4$ 0.04, KI 0.02%.

[4] We are indebted to Mr. E. R. Harding of the H. J. Heinz Company, Pittsburgh, Pennsylvania, and to Mr. C. M. Williams of the Lake Charles Products Corporation, Lake Charles, Louisiana, for supplying, without charge, all the rice cellulose used in this and subsequent studies.

TABLE 2

Levels and ratios of calcium and phosphorus in experimental diets

DIET NO.	PER CENT Ca	PER CENT P	Ca/P RATIO
7	0.49	0.49	1.0
8	0.49	0.735	0.66
10	0.49	2.450	0.2
12	0.735	0.490	1.5
16	1.225	0.245	5.0
19	1.225	1.225	1.0
26	0.017	0.245	0.07
27	0.122	0.245	0.5

the young at 21 days; (2) their percentage ash; and (3) the change in weight of the mother during the reproductive cycle. Owing to the fact that our experiments required the sacrifice of a considerable proportion of the mother rats and their offspring at the time of delivery, we could not employ these

criteria. Moreover, in parathyroid-deficient mothers, milk production was depressed to a point which made it impossible to carry a litter through a 21-day period of lactation successfully. Accordingly, the comparisons have been based on the following considerations: (1) per cent of fertile matings; (2) average number per litter; (3) average weight of rat pups at birth. Data to be submitted in subsequent papers in this series will contribute additional criteria for comparing reproductive success between normal and parathyroid-deficient rats maintained at different levels of intake of calcium and phosphorus.

Per cent of fertile matings. Conforming to the practice at The Wistar Institute (Greenman and Duhring, '31), the rats were mated only after they had attained an age of 110 to 120 days. The routine procedure for breeding in our experiments consisted in accustoming the rat to a particular diet, then exposing the female to its mate for a period of 7 days. Since estrus recurs about every 5 days this assured successful copulation in the majority of instances.

Table 3, column 4, contains comparative data of the per cent of fertile matings in normal and parathyroid-deficient rats kept on various diets. Attention is directed particularly to diets 7, 26, and 27, as these embraced larger numbers of experiments. On diet 7, which Cox and Imboden found to be conducive to maximum reproductive success in normal rats, thirty-five animals were mated a total of 107 times; 65 of the matings proved fertile. The per cent of fertile matings (60.1) was lower than that (76.8) recorded by Cox and Imboden for this diet. The difference may be explained partly on the basis that Cox and Imboden used rats which had previously gone through one successful mating on stock diet. Out of the thirty-five rats on diet 7 in our series, three proved sterile after repeated matings. If correction were made by excluding these three animals, the percentage of fertile matings in this group would be raised to 67%. On diet 26 the percentage fertile matings was 46.7, compared to 50.0% obtained by Cox and Imboden. In thirteen normal rats on diet 27, the per cent fertile matings was 81.5, compared to 68.4% found by Cox and

TABLE 3

Reproductive success in normal and parathyroid-deficient (P-D) rats on various diets

DIET NO.	NORMAL CONTROLS, OR PARATHYROID-DEFICIENT (P-D)	NUMBER IN GROUP	FERTILE MATINGS	NUMBER PER LITTER Min.	Max.	Averages	S.D.[4]	WEIGHTS OF LITTERS Min.	Max.	Averages	S.D.[4]	AVERAGE WEIGHTS OF RAT PUPS Min.	Max.	Averages	S.D.[4]	INDICES OF REPRODUCTIVE SUCCESS IN P-D RATS IN RELATION TO CORRESPONDING CONTROLS
			%					gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
7	Normal	35	65/107 = 60.1 (76.8)[1]	3	16	9.3 (30)[2]	3.1	16.9	76.2	49.62	15.38	4.03	7.10	5.45	0.67	
	P-D	58	55/144 = 38.2	4	13	8.6 (40)	2.3	14.7	54.01	29.19	8.20	1.98	5.38	3.49	0.83	0.733
8	Normal	4	10/10 = 100 (79.2)	10	15	11.5 (6)	1.9	40.00	66.6	56.67	9.43	4.00	5.74	4.95	0.67	
	P-D	4	6/19 = 31.6	7	8	7.5 (2)	0.7	26.5	41.2	33.87	10.38	3.31	5.88	4.58	1.89	0.664
10	Normal	6	14/20 = 70 (71.4)	6	13	10.0 (7)	2.6	27.96	62.53	48.85	12.97	4.66	5.28	4.88	0.23	
	P-D	6	1/24 = 4.2			9.0 (1)				20.34				2.26		0.475
12	Normal	6	12/13 = 92.3 (84.6)	7	13	9.9 (7)	2.3	43.05	72.22	55.47	11.65	4.94	6.43	5.69	0.60	
	P-D	10	15/31 = 48.4	6	11	8.8 (6)	1.7	28.44	53.60	40.01	19.82	3.50	5.36	4.53	0.59	0.736
16	Normal	9	17/26 = 65.4 (70.6)	4	12	8.2 (13)	2.0	23.56	63.72	42.62	9.67	4.4	6.0	5.24	0.49	
	P-D	18	30/55 = 54.5	5	12	9.4 (15)	2.8	26.91	59.40	41.60	9.14	3.0	5.75	4.62	0.72	0.913
19	Normal	5	7/7 = 100 (82.4)	5	11	10.7 (7)	1.6	19.4	55.66	55.47	7.65	4.78	5.87	5.19	0.36	
	P-D	7	5/6 = 83	8	13	9.2 (5)	2.5	41.2	64.5	31.86	14.66	1.86	5.06	3.50	1.15	0.788
26	Normal	16	28/60 = 46.7 (50.0)	5	14	9.5 (14)	1.5	13.65	75.6	45.12	16.96	2.73	6.15	5.14	0.98	
	Mated on 7[3] P-D	6	10/11 = 90.9	8	12	10.0 (10)	1.4	40.00	68.88	52.40	8.02	4.9	5.74	5.24	0.30	
	P-D	23	3/90 = 3.3			4.0 (1)				20.61				5.15		0.495
	Mated on 7[3]	12	12/49 = 24.5	2	14	7.6 (10)	3.9	8.84	35.56	23.24	9.85	2.14	4.68	3.36	0.96	0.557
27	Normal	13	22/27 = 81.5 (68.4)	8	15	11.5 (13)	2.3	40.00	75.48	56.15	11.21	3.05	6.29	4.95	0.87	
	P-D	17	27/92 = 29.35	2	12	6.4 (12)	3.1	10.94	31.00	21.01	6.45	2.13	5.88	3.67	1.11	0.552

[1] Figures enclosed by parentheses in this column denote the values for per cent fertile matings obtained with similar diets by Cox and Imboden ('36a).

[2] Figures in parentheses in this column denote the number of litters on which the averages were based.

[3] Because of inability of parathyroid-deficient rats to breed on diet no. 26, twelve such animals and six controls were mated on diet no. 7, then transferred to diet no. 26, on which they were kept until the conclusion of the gestation period.

[4] S.D. = standard deviation.

Imboden. On diets nos. 10, 12, and 16 there was even closer agreement between our results and those of Cox and Imboden. On diets 8 (four rats, ten matings) and 19 (five rats, seven matings), the percentage fertility in the normal, unoperated rats was, in both instances, 100%.

That parathyroid deficiency is accompanied by marked reduction in fertility is evident from the data in table 3. This was much more conspicuous on certain diets than on others. Thus, in the parathyroid-deficient group on low-calcium diet no. 26, out of ninety matings in twenty-five animals, there were only three known pregnancies (3.3%). However, when parathyroid-deficient rats which after repeated matings had proved sterile on diet no. 26 were changed to some other diet, such as no. 16 or no. 7, a significant number became pregnant. To illustrate, one such group of twelve rats, repeatedly though unsuccessfully mated on diet no. 26, were bred on diet no. 7, then restored to diet no. 26, on which they were kept for the greater part (14 to 19 days) of the gestation period. In this way the percentage of fertile matings was increased to 24.5. The highest incidence of fertile matings in the parathyroid-deficient group, 54.5%, occurred on diet no. 16 (Ca, 1.225%; P, 0.245%; Ca/P ratio, 5.0). The lowest, 3.3%, occurred on diet no. 26 (Ca, 0.017%; P, 0.245%; Ca/P ratio, 0.07). The second lowest, 4.2%, occurred on diet no. 10 (Ca, 0.49%; P, 2.45%; Ca/P ratio, 0.2).

Average number per litter. Greenman and Duhring ('31), describing the experience at The Wistar Institute, state that the number of young per litter varies greatly, but that under favorable conditions the average may reach seven or eight, or even more. The average for first litters is given as 9.97, that for fifth litters as 6.0. Reference to table 3 shows that for the unoperated rats the average number per litter varied from 8.2 on diet no. 16 to 11.5 on diets 8 and 27. For the parathyroid-ectomized rats the averages were distinctly lower than those for the normals on all diets excepting 16. Although small litters occurred somewhat more frequently among the para-thyroid-deficient rats than among the normals, the range of

variation in the numbers per litter was not strikingly different in the two groups of animals.

Average weight of rat pups at birth. Several factors are known to influence the birth weight of rats. Increased maternal age and weight are associated with heavier birth weights. Newborn males weigh on the average 0.3 gm. more than females. Increase in the number per litter tends to diminish the birth weight. The nutritional status of the mother is also important, an inadequate or unbalanced diet being associated with low birth weight. Season may be a factor, as shown by statistics obtained at The Wistar Institute (Greenman and Duhring, '31).

The data for normal birth weights recorded in the literature show considerable variation. Thus, the majority of the values cited by Donaldson ('24) are below 5 gm., while those given by Greenman and Duhring ('31) range from 5.33 to 5.84 gm. for females and from 5.6 to 5.95 gm. for males. Jackson ('40) recently reported his experience with two diets. One group of ten female rats, kept on a commercial dog food,[2] produced forty-two litters, averaging 9.93 rats per litter, with an average birth weight of 5.20 gm. A second group of ten rats, kept on McCollum diet 1, produced thirty-eight litters, averaging 8.21 rats per litter, with an average birth weight of 5.23 gm. Cox and Imboden ('36 a) give 5.37 gm. as the average birth weight of 239 first-litter rats, with the mothers kept on a stock diet, and 5.12 gm. for second-litter rats on various experimental diets. In our experiments the birth weights of the young from normal mothers kept on diet no. 7 (279 pups) averaged 5.45 gm. The highest birth weights, average of 7.10 gm., occurred in a litter of three (a fifth litter); the lowest average value, 4.03 gm., occurred in a first litter of twelve. The largest litter on diet 7, a first litter, consisted of sixteen young with an average weight of 4.76 gm.

Diet seemed to be a much more significant factor in the parathyroid-deficient group than in the normals. In the normal controls the lowest average birth weight, 4.88 gm., was ob-

[2] Purina dog chow.

tained on diet no. 10 (seventy young); the highest average value for the whole group on any diet, 5.69 gm., occurred on diet no. 12 (seventy young). In the parathyroid-deficient group the lowest average value, 2.26 gm., was obtained on diet no. 10 (one litter of nine), while the highest value, 4.62 gm., occurred on diet no. 16 (128 young). On diet no. 12, the average birth weight in the parathyroid-deficient group was 4.53. From the data in table 3 it is apparent that parathyroid deficiency in the mothers produced retardation of fetal growth and that this effect was more marked on some diets than on others.

INDICES OF REPRODUCTIVE SUCCESS

The ratio: $\frac{\text{per cent fertile matings in parathyroidectomized rats}}{\text{per cent fertile matings in normal controls}}$ varied from 0.139 on diet no. 26 to 0.834 on diet no. 16. The corresponding ratios for the average number per litter varied from 0.556 on diet no. 27 to 1.023 on diet no. 16, while the ratios for the average birth weight varied from 0.463 on diet no. 10 to 0.926 on diet no. 8. The sum of the ratios for any particular group divided by 3 provides an index which may be used for rough comparisons of reproductive success on the various diets employed in the study (see table 3). The lowest index was obtained on (high phosphorus) diet no. 10, 0.475, and the highest on (high calcium-low phosphorus) diet no. 16, 0.913. It is of interest that the latter diet, though rachitogenic and therefore unsatisfactory under ordinary circumstances, was singularly effective in promoting reproductive success in the parathyroid-deficient rat.

However, neither the individual factors considered so far nor the indices just mentioned describe adequately the effects of hypoparathyroidism on reproduction. Long ago, Halsted (1896) noted that partly "thyroidectomized" dogs which had become pregnant developed tremors and convulsions at term. Carlson ('13) found that thyro-parathyroidectomy in dogs in the latter stages of pregnancy resulted in a high mortality. Thus, eight out of fifteen animals died in 12 to 24 hours after the operation. The effects of parathyroid deficiency in pregnant animals have been recorded also by Luckhardt and

Rosenbloom ('22), by Dragstedt, Sudan and Phillips ('24), Kozelka, Hart and Bohstedt ('33) and by others. Bodansky and Cooke ('37) reported the results of thyro-parathyroidectomy in rats. Out of sixty-two animals, twenty-four succumbed at the conclusion of the first pregnancy, although they had appeared in normal health until 2 or 3 days before term. Eighteen others did not survive subsequent pregnancies. That the symptoms developing at term were due largely to parathyroid deficiency has been established by extending the studies to rats deprived of the parathyroids only.

The course of pregnancy in the parathyroidectomized rat has been summarized previously by Bodansky and Duff ('39). When kept on satisfactory rations these animals may become pregnant and thrive until toward the end of gestation. However, from 2 to 4 days before term slight tremors of the paws develop, these being more apparent when the rat is held up. The tremors become progressively more pronounced, the body is stiffened and the tail becomes rigid. The rat moves stiffly and with apparent difficulty even on a flat surface. Anorexia is a prominent symptom. Despite these severe manifestations the rat may improve, go through delivery and recover. Or, the downward course may proceed in one of two ways. Rarely, the tremors become more and more severe, ultimately assuming tetanic convulsive proportions; the rat loses consciousness and death occurs frequently within about 12 to 18 hours after onset of symptoms. Much more commonly the tremors cease; the rat becomes more and more quiescent and then lethargic. It can be roused only by pronounced stimuli. Meanwhile the body temperature drops, and finally the rat dies in coma.

These events may commence after the onset of labor (appearance of blood at the vagina). As a rule death occurs before any of the young have been delivered, but occasionally it may follow the delivery of part of the litter. A few of the animals in our series died after completing delivery, but in these, labor was markedly prolonged (from 1 to 2 days). In parathyroid-deficient rats the onset of labor is frequently de-

layed beyond the normal gestation period. As a rule the duration of labor is also prolonged.

Such manifestations have not been observed among the unoperated control rats, or among the stock animals in our colony.

Serum calcium. The relation of the symptoms to the low serum calcium at term has been investigated (table 4). In the control rats fed diet no. 7 the serum calcium during parturition averaged 9.0 ± 1.09 mg., whereas in the parathyroidectomized animals on the same diet, the average was 4.65 ± 1.06. Similar relations held for other diets, excepting (high calcium) diet no. 16. On this diet, which was clearly rachitogenic (Ca/P ratio = 5), the serum calcium of the parathyroid-deficient rats during labor varied from 7.85 to 14.3 mg., the average being 11.50 ± 1.88. This was only slightly below the average of 11.77 ± 1.51 recorded for the control animals on the same diet. It may be noted further that this was the only diet in our series which did not affect adversely the course of pregnancy and parturition in parathyroidectomized rats. That this was related to the relatively high calcium intake and the maintenance of a normal level of calcium in the blood was demonstrated as follows. Rats which had successfully completed one or more pregnancies on diet no. 16, and which were shown to have normal serum calcium during labor, were transferred to diet no. 7. Invariably the succeeding pregnancy ended abnormally; the rat developed the characteristic symptoms of parathyroid deficiency and in those instances in which blood was obtained for analysis, the serum calcium was found invariably to be low (5.2 mg. per 100 cc., or less).

The lowest serum calcium, 1.94 mg. per 100 cc., occurred during labor in a parathyroidectomized rat on diet no. 27.

Inorganic phosphorus. The concentrations of phosphorus in the blood serum showed wide variation but were, in general, higher in the parathyroid deficient rats than in the controls. Thus, in the unoperated rats fed diet no. 7 the values varied

TABLE 4

Serum calcium, phosphorus and phosphatase of normal and parathyroid-deficient pregnant rats *at term*

NORMAL OR PARATHYROID-DEFICIENT (P-D)	DIET NO.	CALCIUM IN MG. PER 100 CC.					INORGANIC P IN MG PER 100 CC.					PHOSPHATASE UNITS				
		Analyses	Min	Max	Averages	S.D.[2]	Analyses	Min	Max	Averages	S.D.[2]	Analyses	Min.	Max.	Averages	S.D.[2]
Normal	7	27	7.76	11.00	9.10	1.09	25	2.6	8.0	4.67	1.64	23	3.54	36.7	17.4	9.93
P-D		39	2.37	7.00	4.65	1.06	31	2.8	27.8	7.81	4.77	26	3.64	30.6	9.19	5.22
Normal	8	5	7.5	10.6	8.80	1.13	5	3.95	11.2	6.07	2.94	5	11.64	26.8	21.64	5.93
P-D		2	3.1	3.7	3.40	0.42	1	—	—	8.0	—	1	—	—	6.8	—
Normal	10	6	7.2	12.2	9.88	1.76	5	4.38	6.0	4.97	0.74	5	12.3	35.7	23.7	9.52
P-D		1	—	—	3.20	—	1	—	—	5.25	—	1	—	—	15.2	—
Normal	12	8	7.6	11.14	9.36	1.06	7	2.45	10.37	5.66	2.86	7	8.07	38.1	24.78	9.70
P-D		6	3.4	5.3	4.5	0.73	4	2.50	10.10	7.39	3.53	4	2.67	23.5	11.94	9.12
Normal	16	8	10.0	15.0	11.77	1.51	6	0.55	5.74	2.52	1.74	6	7.16	35.25	13.30	10.84
P-D		13	7.85	14.3	11.50	1.88	8	1.28	2.49	1.90	0.55	8	4.22	30.10	13.08	9.34
Normal	19	5	5.35	9.16	8.09	3.16	5	4.26	6.18	5.26	0.74	5	8.03	14.08	10.37	2.36
P-D		4	3.27	4.98	3.85	0.77	4	4.97	12.67	8.34	3.19	2	5.21	6.03	5.62	0.58
Normal	26	17	4.7	10.80	8.18	1.86	16	1.85	7.50	3.90	1.08	13	2.48	22.08	12.58	5.95
P-D [1]		10	2.85	5.33	4.06	0.79	9	3.03	7.23	5.44	1.31	9	4.22	27.50	10.31	7.36
Normal	27	12	7.07	11.55	9.14	1.49	10	2.65	6.56	5.13	1.16	9	5.76	27.16	20.45	9.22
P-D		11	1.94	6.00	4.24	1.18	11	5.92	19.7	10.76	4.59	9	2.72	30.25	14.89	8.71

[1] Because of inability of parathyroid-deficient rats to breed on diet no. 26, nine of these animals were mated on diet no. 7, then transferred to diet no. 26 on which they remained for a period varying from 14 to 19 days, until the conclusion of gestation.

[2] S.D. = standard deviation.

from 2.6 to 8.0 mg. (average 4.67 mg.) per 100 cc., while in the parathyroidectomized rats on the same diet the values varied from 2.8 to 27.8 mg. (average 7.81 mg.) per 100 cc. These observations are in general agreement with the accepted view (Greenwald and Gross, '25; Logan, '39, and others) that the parathyroid hormone regulates the excretion of inorganic phosphate and therefore affects its concentration in the blood. The lowest values for serum inorganic phosphorus were observed on diet no. 16 (Ca, 1.225%; P, 0.245%). The high Ca/P ratio was evidently a factor in maintaining the serum inorganic phosphorus at a low level not only in the normal controls, but in the parathyroid-deficient rats as well.

Serum phosphatase. The phosphatase activity of the serum, and more particularly the phosphatase of osseous origin, is believed to be an expression of the specific reactivity of the bone (Bodansky and Jaffe, '34). From the data in table 4 we note that the phosphatase varied over a wide range in each group, but that the average values were, with the exception of the rats on diet no. 16, higher in the normal controls than in the operated rats. Owing to the variability of the data in different individuals of the same group, the probable significance of single results cannot be defined with certainty. However, the data indicate that in general the capacity for cellular activity in bone tended to be less in the parathyroid-deficient rats than in the normal animals, a conclusion which is in line with existing knowledge of the relation of the parathyroids to bone metabolism.

It is interesting to note that diet no. 16, owing to its high calcium-phosphorus ratio, is definitely rachitogenic and thus promotes demineralization of the maternal skeleton and prevents normal fetal calcification. At the same time, this diet is effective in abolishing the discrepancies between normal and parathyroidectomized pregnant rats with respect to serum calcium, phosphorus and phosphatase.

SUMMARY

Parathyroid deficiency in the rat is associated with markedly diminished fertility, moderate reduction in the number per litter and a significant reduction in the average birth weight. These effects are more conspicuous on diets either low in calcium or high in phosphorus.

Parathyroid deficiency in the pregnant rat is also characterized by prolongation of the gestation period, tetany at term, prolonged labor and high rates of maternal and fetal mortality. These abnormal manifestations appear to be related to the marked lowering in serum calcium.

The concentration of inorganic phosphate in the blood serum of operated animals is usually above that of the controls on the same diet. The phosphatase is usually lower than in the controls, indicating that the capacity for cellular activity is less in the bones of the parathyroid-deficient rat than in those of the normal animal.

In parathyroid-deficient rats fed a diet abnormally high in calcium and relatively low in phosphorus (diet no. 16 of Cox and Imboden), the values for serum calcium, phosphorus and phosphatase fell within the same limits of variation and showed approximately the same averages as in the normal controls. The abnormal manifestations of late pregnancy were likewise abolished and the differences in per cent fertility, average number per litter and average birth weight were greatly minimized.

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THE DISTRIBUTION OF PANTOTHENIC ACID IN CERTAIN PRODUCTS OF NATURAL ORIGIN

THOMAS H. JUKES

Division of Poultry Husbandry, University of California, Davis

The distribution of pantothenic acid in a number of foods was reported previously in several communications (Jukes and Lepkovsky, '36; Jukes, '37; Jukes and Richardson, '38) in terms of "filtrate factor units" as determined by assay with chicks. Subsequently it was observed that one such "unit" corresponded to 14 micrograms of pantothenic acid (Jukes, '39). The present report summarizes the results of biological assays of various materials by the chick method previously described (Jukes, '37).

The use of chicks for the assay is cumbersome by comparison with microbiological methods (Pennington, Snell and Williams, '40; Strong, Feeney and Earle, '40). However, from the standpoint of nutrition, the chick assay method is perhaps advantageous in that it parallels the conditions encountered during alimentation. These observations do not alter the probability that the microbiological assay method will almost entirely replace the chick assay method in the future.

EXPERIMENTAL

The basal diet (80G) was previously described (Jukes, '39). In recent assays, the "positive control diet" consisted of 100 gm. of diet 80G plus 6 mg. of synthetic (dl) pantothenic acid. It has been shown (Babcock and Jukes, '40) that synthetic (dl) pantothenic acid has one-half the activity for chicks of that possessed by natural pantothenic acid. The entire biological activity of synthetic pantothenic acid has been

shown by Stiller and co-workers ('40) to be due to the dextro-rotatory isomer. One chick "filtrate factor" unit therefore corresponds to 14 µg. of dextro-rotatory pantothenic acid and the levo-rotatory form is without biological activity. The level of synthetic pantothenic acid fed in the positive control diet was slightly more than twice the amount necessary for maximum growth. Assay diets were supplemented when possible with levels of the test material sufficient to produce between 60 and 80% of the maximum growth response.

The care of chicks has been described previously (Jukes, '37). Following hatching, the chicks were kept on a "normal" diet for 1 week. The "normal" diet used was Chick Mash 1 (Almquist, Jukes and Newlon, '38). Following this, diet 80G was fed for a depletion period of about 2 weeks. The chicks were then weighed, divided into weight classes, separated into groups, and fed the test diets for an assay period of 9 to 14 days. The assay was usually terminated by the time the positive control group had gained an average of from 60 to 90 gm.

The calculation of the pantothenic acid content of the supplements was explained previously (Jukes and Lepkovsky, '36) and is as follows:

Pantothenic acid content of supplement in micrograms per gram =

$$\frac{(\text{Gain on test diet } minus \text{ gain on basal diet}) \times 14}{(\text{Gain on positive control diet } minus \text{ gain on basal diet}) \times (\text{Amount of supplement in 1 gm. of test diet})}$$

This revised calculation is based on the finding that one chick "filtrate factor" unit corresponds to 14 µg. of natural pantothenic acid (Jukes, '39). Similar results for the conversion factor have been obtained with synthetic pantothenic acid. Supplements to be tested were incorporated with the heated diet as explained previously (Jukes, '39). Foods containing large amounts of water were mixed with the diet and passed through a food-chopper or rubbed through a sieve. The mixture was then dried in thin layers at room temperature and re-weighed.

The data from a typical assay are presented in table 1.

From table 1 it may be calculated that the pantothenic acid contents of dried carrots and dried broccoli are respectively 13 and 46 µg. per gram.

The results of a number of assays are presented in table 2. Also included are some previously published data which have been recalculated in terms of micrograms of pantothenic acid per gram of supplement. The materials tested are divided into arbitrary classes with respect to pantothenic acid content. For classification, the pantothenic acid content is expressed in micrograms per gram of dry material. Many of the foods

TABLE 1

Chicks hatched and placed on normal diet on June 6. Placed on heated diet on June 13. Weighed and segregated into groups of eight on June 26

GROUP	SUPPLEMENT TO 100 GM. DIET 80G	WEIGHT IN GRAMS			NUMBER OF CASES OF DERMATITIS	
		June 26	June 30	July 6	June 26	July 6
1	None	85	88	90	3	7
2	19 gm. dried carrots	83	91	102	3	5
3	17 gm. dried broccoli	86	106	135	3	0
4	6 mg. racemic pantothenic acid	85	114	168	4	0

tested were high in water. These are expressed in terms both of fresh material and dry material. Other products, such as grains and nuts, are expressed in terms of the undried materials.

DISCUSSION

Wide variation was found to exist in the pantothenic acid content of various samples of the same product. For this reason, results in table 2 which are based on small numbers of assays may be less reliable than results in which larger numbers of assays were involved. In some instances, such as eggs, the variation may be due to the diet of the animal concerned (Lepkovsky et al., '38). The cereal grains appeared to be less variable than a number of the other products. Most of the fruits tested were poor sources.

TABLE 2

Distribution of pantothenic acid in certain products of natural origin, in terms of micrograms of pantothenic acid per gram of material. All common human foods are expressed on the basis of the edible portion

MATERIAL	PANTOTHENIC ACID CONTENT IN MICROGRAMS PER GRAM				NUMBER OF ASSAYS
	Dry material		Undried basis		
	Range	Average	Range	Average	
Excellent sources (more than 28 micrograms per gram of dry material)					
Brewers' dried yeast	140 to 350	200			10
Liver, mammalian	100 to 270	180	25 to 60	40	4
Egg yolk	100 to 200	125	50 to 100	63	4
Eggs	32 to 190	108	8 to 48	27	37
Commercial "liver meal"			100 to 110	105	2
Broccoli leaves and blossoms		87		14	1
Cane molasses, "black strap"	5 to 100	70			11
Peanut meal			45 to 63	53	3
Whey	36 to 85	60	2.4 to 5.7	4	17
Broccoli		46		11	1
Buttermilk, churned	35 to 56	46	3.5 to 5.6	4.6	4
Sweet potatoes	31 to 46	38	10 to 12	11	2
Lean beef		38		10 ·	1
Skim milk	21 to 43	36	2.1 to 4.3	3.6	9
Zucchini squash		35		3	1
Kale	23 to 36	30	2.3 to 3.6	3	2
Good sources (14 to 28 micrograms per gram of dry material)					
Canned salmon		28		7	2
Irish potatoes		28		6.5	2
Taro root		28		7	1
Alfalfa	3 to 40	25			12
Wheat bran			20 to 30	24	4
Canned pumpkin		23		4	1
Whole milk	10 to 32	22	1.3 to 4.2	2.8	4
Rice bran			15 to 27	22	3
Split peas			20 to 22	21	2
Tomatoes		20		1	2
Cowpeas			17 to 20	18	2
Soybean meal			8 to 22	14	4
Cottonseed meal				14	2
Moderate sources (10 to 14 micrograms per gram of dry material)					
Carrots		13		2	1
Jerusalem artichoke		13		4	1
Rolled oats				11	2
Wheat				11	2
Wheat middlings			10 to 11	10	2
Barley				10	2

TABLE 2 — *continued*

MATERIAL	PANTOTHENIC ACID CONTENT IN MICROGRAMS PER GRAM				NUMBER OF ASSAYS
	Dry material		Undried basis		
	Range	Average	Range	Average	
Poor sources (4 to 10 micrograms per gram)					
Spinach		10		1.2	2
Milo				9	2
Onion	8 to 11	9	1 to 1.4	1.2	2
Yellow corn			7 to 10	8	3
English walnuts				8	1
Wheat germ			3 to 12	7	4
Orange		7		0.7	2
Sesame meal				6	2
Polished rice				4	2
Banana		4		0.7	2

Other moderate to poor sources, all giving negative results in the chick assay at the level fed

SOURCE	LEVEL FED (GM. DRY FOOD PER 100 GM. DIET)	UNDRIED EQUIVA-LENT	PANTOTHENIC ACID CONTENT, MICROGM. PER GM. ON DRY BASIS (EXCEPT [1])
Ladino clover	8	51	Less than 1.2
Blood meal		8	,, ,, 1.2 [1]
Canned green beans	9	80	,, ,, 1.1
Egg white	9	80	,, ,, 1.1
Linseed meal		10	,, ,, 1.0 [1]
Coconut meal		10	,, ,, 1.0 [1]
Barley shoots	10	88	,, ,, 1.0
Lawn clippings	10	40	,, ,, 1.0
Broccoli stems	12	89	,, ,, 0.9
Asparagus	12	89	,, ,, 0.9
Commercial casein		12	,, ,, 0.9 [1]
White turnips	13	130	,, ,, 0.8
Canned green peas	14	54	,, ,, 0.7
Prunes		20	,, ,, 0.6 [1]
Raisins		20	,, ,, 0.6 [1]
Fish meal		20	,, ,, 0.6 [1]
Meat scrap		20	,, ,, 0.6 [1]
Canned peaches	23	156	,, ,, 0.5
Hempseed meal		25	,, ,, 0.4 [1]
Canned pineapple juice	26	148	,, ,, 0.4
Beets	27	141	,, ,, 0.4
Apples	32	195	,, ,, 0.3
Almonds		40	,, ,, 0.3 [1]

[1] Calculated on undried basis.

Several water-soluble vitamins, namely, thiamin, riboflavin, nicotinic acid, pyridoxine and pantothenic acid, are grouped under the term "vitamin B complex." It has long been recognized that the members of this group are commonly associated with each other in nature, and in recent years the discovery of certain of the biochemical functions of these vitamins has made their widespread occurrence in living tissues readily understandable. It is important to recognize, however, that the relative proportions of the B-complex vitamins differ widely in various foods. This observation may play a part in the "balancing" of diets. The following data are illustrative:

TABLE 3

Illustrating the variation in the distribution of certain members of the vitamin B complex as compared with the nutritional requirements of chicks

	PARTS PER MILLION		
	Thiamin	Riboflavin	Pantothenic acid
Chick requirement in diet	1.5	2.7	14
Egg (dry basis)	4	12	108
Liver (dry basis)	12	100	180
Cow's milk (dry basis)	2.5	18	22
Wheat	5	1	11
Wheat bran	6	4	24
Wheat germ	40	8	7

For example, from table 3 it may be seen that egg, liver or milk will supply the dietary requirement of the chick for thiamin, riboflavin and pantothenic acid. The three cereal products listed will supply the thiamin requirement but in some instances are deficient in riboflavin or pantothenic acid. Comparatively large amounts of riboflavin and especially pantothenic acid are present in two foods, egg and milk, which are intended for the nutrition of young animals. The data in table 3 for the thiamin content of the foods listed were obtained from recent literature on the subject. The other figures were from experimental results in this laboratory.

The question of secondary deficiencies in diet 80-G is of importance in the assay method. Observations in this labora-

tory have indicated that pantothenic acid, when added to the diet at a level of 14 or more parts per million of diet, will produce a growth response in the test as great as that produced by crude sources of pantothenic acid, such as rice bran filtrate, when these sources are added to the diet at the level sufficient for maximal growth. This does not exclude the possible existence of other nutritional essentials in rice bran and other filtrates. The crude materials, such as commercial casein and whey adsorbate, present in diet 80-G are thought to provide some of these unknown factors. The whey adsorbate should supply at least 400 µg. of riboflavin per 100 gm. of diet. The above conclusions are not necessarily valid if changes are made in the heated diet, or if prolonged assay periods are used. An assay period of from 7 to 9 days is usually satisfactory, although 14 days has been used in many of the assays reported in table 2, especially in the case of the earlier data. Recent results have indicated that the method gives reasonable agreement with the lactobacillus casei assay method when applied to crude materials.

SUMMARY

1. The pantothenic acid content of a number of foods and other products of natural origin was measured by biological assay with chicks. Data are presented for sixty-eight products, involving more than 200 bio-assays.

2. The dietary requirement of the chick for thiamin, riboflavin, and pantothenic acid is compared with the varying distribution of these vitamins in certain foods.

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THE EFFECT OF DIET ON THE PANTOTHENIC ACID CONTENT OF EGGS[1]

ESMOND E. SNELL, ESTHER ALINE, J. RUSSELL COUCH AND
PAUL B. PEARSON

*Department of Chemistry, University of Texas, Austin, and the Agricultural
Experiment Station, Agricultural and Mechanical College of Texas,
College Station*

While it is well established that the pantothenic acid content of the tissues of growing chicks decreases when they are restricted to a diet deficient in this constituent (Snell et al., '40) evidence on the influence of the amount in the diet on the level in the eggs is limited and inconclusive. Lepkovsky et al. ('38) reported that pantothenic acid, under the conditions studied, appears to exercise no function in maintaining normal egg production. On the other hand Bauernfeind and Norris ('39) presented evidence that pantothenic acid is necessary for normal hatchability. On the basis of hatchability data, they concluded that the pantothenic acid content of eggs can be influenced by the amount in the diet.

Since more refined methods for the determination of pantothenic acid (Pennington et al., '40) are now available it was of interest to determine if and to what extent the pantothenic acid content of the diet influences the amount in the eggs.

EXPERIMENTAL

For the data on eggs from hens receiving a deficient diet, four White Leghorn hens approximately 8 months of age were used. These birds had received since hatching a diet consisting of yellow corn 74.5, purified casein 18, salts mixture 7.5,

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[1] Published with the approval of the Director of the Texas Agricultural Experiment Station as technical contribution no. 616.

riboflavin 250 μg. per 100 gm. of diet, and cod liver oil to furnish 900 U.S.P. units of vitamin A and 150 A.O.A.C. chick units of vitamin D per 100 gm. of diet. Chicks fed this diet developed the typical dermatitis from which they recovered when the diet was supplemented with a crude preparation of pantothenic acid at a rate calculated to furnish 1.4 mg. of the pure acid per 100 gm. The four hens were among those that survived the deficiency and eventually reached approximately normal size and appearance. Their egg production during the 3 months preceding these studies, while somewhat irregular, had at various times reached as many as five to six eggs per week.

The normal eggs were from hens fed a stock diet having a percentage composition of milo 40, oats 10, wheat shorts 20, fish meal 6, cottonseed meal 6, soybean oil meal 6, alfalfa leaf meal 5, dried whey 3, oyster shell 2, bone meal 1, sodium chloride 1, and cod liver oil to furnish 600 U.S.P. units of vitamin A and 80 A.O.A.C. chick units of vitamin D per 100 gm. of diet in addition to that present in the feeds. This diet contained 1.9 mg. of pantothenic acid per 100 gm. as compared with 0.44 mg. for the deficient diet.

The pantothenic acid was determined by the bacterial method of Pennington et al. ('40). Four eggs were collected from each of the hens on the deficient diet and analyses made on the combined whites and yolks from two eggs. Since the values obtained on each set of two eggs were in good agreement, only the averages of the two values are recorded in table 1. The data from the hens on the stock diet are the values obtained from the analyses of the combined whites and yolks, respectively, from two eggs.

The data in table 1 show that the amount of pantothenic acid in the eggs is dependent upon the amount in the diet. The whites of the eggs from the hens on the deficient diet contained an average of 0.41 μg. of pantothenic acid per gram and the yolks 9.66 μg. The corresponding figures for the eggs from the hens on the stock diet are 0.76 μg. for the whites and 46.6 μg. per gram of yolk. These figures for normal eggs, though

slightly lower, are of the same order as the values calculated from the data of Jukes ('37) for egg white and yolk on the basis that 1 chick filtrate factor unit corresponds to 14 µg. of pantothenic acid (Jukes, '39). Such differences are not unexpected since as our data show, the amount in the egg is dependent upon the level in the diet.

To study the effect of supplementing the deficient diet with pantothenic acid 8.4 mg. of *dl*-sodium pantothenate (Williams et al., '40) (4.2 mg. of the active isomer: Stiller et al., '40; Babcock and Jukes, '40) was added to each 100 gm. of feed.

TABLE 1

Pantothenic acid content of eggs from hens on basal stock diets

NUMBER	DIET	WHITE	YOLK	WHOLE EGG (WITHOUT SHELL)
		µg./gm.	*µg./gm.*	*µg./100 gm.*
63	Deficient	0.38	11.5	435
64	,,	0.34	7.3	254
65	,,	0.33	7.4	289
66	,,	0.60	12.5	467
Average		0.41	9.66	361
43	Stock	0.91	45.3	1730
45	,,	0.59	35.6	1270
46	,,	0.73	45.5	1896
47	,,	1.00	48.5	1850
48	,,	0.87	68.5	2485
55	,,	0.47	37.5	1170
Average		0.76	46.8	1732

This is three times the daily requirement for growing chicks as recommended by Jukes ('39).

Data are presented in table 2 showing the pantothenic acid content of the whites and yolks of hens on the deficient diet and the values at various periods after the supplement was added.

Weight records of whites and yolks of all eggs were kept, and from these the pantothenic acid content per 100 gm. of egg (white plus yolk, neglecting the shell) was calculated. These values are given in column 5 of tables 1 and 2. The effect of the addendum is immediately reflected by an increase in the pantothenic acid content of both the whites and yolks. This

increase continued for about 18 days after supplementation was begun, then approached an approximately stationary level more than twice that in the eggs from hens on the stock diet. While the ratios of the pantothenic acid contents of the deficient diet to the stock diet to supplemented diet were 1:4.3:10.5, those of the eggs obtained from hens maintained on these diets were 1:4.8:9.8.

TABLE 2

Effect of supplementing deficient diet with pantothenic acid

NUMBER	DAYS ON PANTO-THENIC ACID	PANTOTHENIC ACID		WHOLE EGG (WITHOUT SHELL)
		White	Yolk	
		μg./gm.	μg./gm.	μg./100 gm.
64	Initial	0.34	7.3	254
	3	0.48	12.0	488
	8	1.90	34.0	1210
	18	2.20	100.0	3320
	24	3.20	92.0	3400
65	Initial	0.33	7.4	289
	4	0.52	11.0	417
	14	3.50	54.0	2070
	18	1.70	98.0	3470
	20	3.60	100.0	3870
66	Initial	0.60	12.5	467
	5	1.40	24.0	935
	6	2.00	27.0	1095
	14	1.90	63.0	3210

Within the limits of errors in sampling, assay, etc., these ratios are identical. It is thus evident that the pantothenic acid content of the egg is directly dependent upon the level at which pantothenic acid is fed.

SUMMARY AND CONCLUSIONS

Eggs from hens maintained since hatching on a diet low in pantothenic acid showed a markedly lowered pantothenic acid content. This increased rapidly when the diet was supplemented with synthetic pantothenic acid. From quantitative data secured, it is concluded that within the limits tested the

pantothenic acid content of the egg is directly proportional to that of the diet.

We wish to express our thanks to the Rockefeller Foundation for grants in partial support of this investigation.

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BRIEF HISTORICAL NOTES

ON

MEAD'S CEREAL AND PABLUM

HAND in hand with pediatric progress, the introduction of Mead's Cereal in 1930 marked a new concept in the function of cereals in the child's dietary. For 150 years before that, since the days of "pap" and "panada," there had been no noteworthy improvement in the nutritive quality of cereals for infant feeding. Cereals were fed principally for their carbohydrate content.

The formula of Mead's Cereal was designed to supplement the baby's diet in minerals and vitamins, especially iron and B_1. How well it has succeeded in these functions may be seen from two examples:

(1) As little as one-sixth ounce of Mead's Cereal supplies over half of the iron and more than one-fifth of the vitamin B_1 minimum requirements of the 3-months-old bottle-fed baby. (2) One-half ounce of Mead's Cereal furnishes all of the iron and two-thirds of the vitamin B_1 minimum requirements of the 6-months-old breast-fed baby.

That the medical profession has recognized the importance of this contribution is indicated by the fact that cereal is now included in the baby's diet as early as the

third or fourth month instead of at the sixth to twelfth month as was the custom only a decade or two ago.

In 1933 Mead Johnson & Company went a step further, improving the Mead's Cereal mixture by a special process of cooking, which rendered it easily tolerated by the infant and at the same time did away with the need for prolonged cereal cooking in the home. The result is Pablum, an original product which offers all of the nutritional qualities of Mead's Cereal, plus the convenience of thorough scientific cooking.

During the last ten years, these products have been used in a great deal of clinical investigation on various aspects of nutrition, which have been reported in the scientific literature.

Many physicians recognize the pioneer efforts on the part of Mead Johnson & Company by specifying Mead's Cereal and PABLUM.
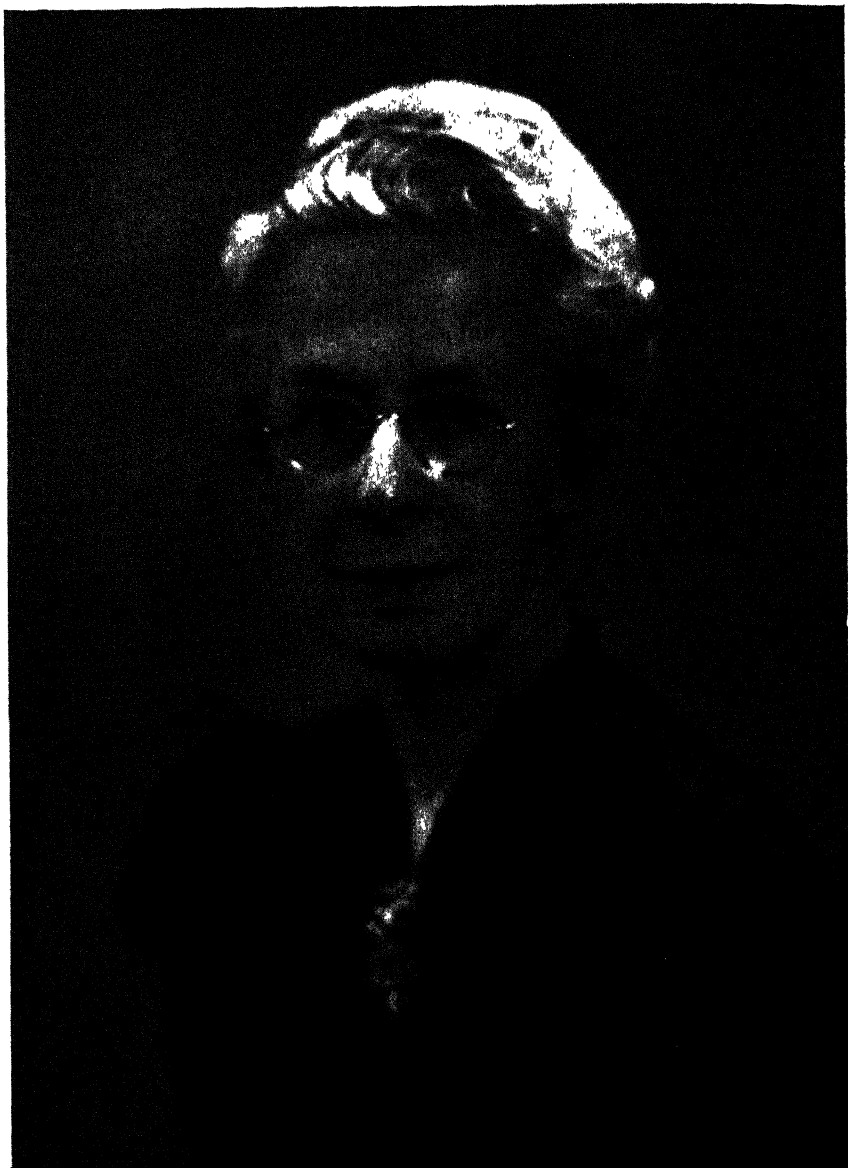
PHOTOGRAPH BY MRS. W BURDEN STAGE

MARY SWARTZ ROSE

1874 – 1941

MARY SWARTZ ROSE

An appreciation

The first of the former presidents of the Institute of Nutrition to pass from us was Professor Mendel, the second is one of his students whom he always regarded with an outstanding esteem, and who bore a distinguished part in the carrying on of his tradition and ideals, Doctor Mary Swartz Rose.

If in her case the doctoral rather than the professorial title comes first to mind, it is partly because she wore the former longer; and also because she exemplified so perfectly the definition of what the modern doctorate should mean—''a broadly educated person, sharpened to a point.''

Only after a classical education in the liberal arts (Litt.B., Denison, 1901), did she turn her attention predominantly to science, and then with characteristic breadth of view she passed from the literary course of a classical college to the technical training of The Mechanics' Institute. Later, two years as student and assistant at Teachers College (B.S., 1906) with further study of food and nutrition in the Columbia department of chemistry, resulted in her definite, mature decision to make the science and teaching of nutrition her life work. To perfect her preparation for such a career she studied two years with Professor Mendel at Yale, receiving its Ph.D. degree in 1909.

Directly upon the completion of her work with Doctor Mendel she was appointed instructor in Teachers College, Columbia University, and became its first faculty member to devote full time to the teaching of nutrition and dietetics. The time was ripe for the development of this field, especially in the education of those who would teach nutrition and dietetics in the rapidly growing departments and schools of home economics

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in colleges and universities. Her success was immediate, and the rapid growth of her reputation was reflected in her successive promotions through the academic grades to the position of professor of nutrition; and in the numbers of exceptionally able people who came to study with her, and the eagerness with which they were sought for appointment to professorships in all parts of our continent and in many other parts of the world. She followed the tradition of Doctor Mendel's seminar method in her own; with its broad scientific scholarship and historical perspective in the arrangement of the readings, the discrimination with which they were assigned, and the skill with which the findings were finally summarized.

As a classroom and laboratory teacher of unified courses in nutrition and dietetics at the college and graduate levels she brought to bear her scientific and technical training and her life-long devotion to the art of teaching. Unquestionably a gifted teacher, she never permitted herself to rest upon the consciousness of her gift. She made her career an unceasing self-discipline, and always unreservedly and with keen alertness, she gave of her best in her daily work. The clarity and cogency of her exposition were the result of constant study and of systematic preparation. In addition she spoke with an infectious sincerity which inspired her students to be disciples and loyal coworkers.

Her knowledge and skill she gave also to the carrying of the new science of nutrition directly into public service; and on all fronts. Her public lectures and her writings—especially "Foundations of Nutrition" and "Feeding the Family" which so outstandingly combine interest, practicality, and scientific soundness—linked the findings of the nutrition laboratory with the daily lives of the people. Generously too, she joined in team-work for the further extension of nutritional knowledge and service through civic agencies and professional organizations—local, national, and international. She carried the message of what nutrition can mean for health and welfare into the public schools and the nursing and health centers of her community; she was long a member of the editorial board of The Journal of Nutrition, and was president of the Institute of Nutrition in 1937–1938; she served as a member of the

Council on Foods and Nutrition of the American Medical Association; and of the nutrition committee of the League of Nations. She was chosen by the international quarterly "Nutrition Abstracts and Reviews" to write a comprehensive account and interpretation of the college and university teaching of nutrition and dietetics in the United States. She served as deputy director of the bureau of conservation of the Food Administration in 1918–1919; and in 1940 was chosen one of a national group of five to serve as advisors on nutrition to the Council of National Defence, and consultants to the committee on food and nutrition of the National Research Council. These are but examples of her far-reaching service.

Her combination of breadth of view, depth of insight, and unsparing concentration of herself upon the effort in hand, is shown also in her record of research. Her versatility and wide reading are reflected in the range of her research topics; and their timeliness reflected the judgment of a critical and creative teacher as to what, at a given period, was most needed for the symmetrically strong development of our rapidly expanding science. Her critically constructive function in relation to the science of nutrition as a whole gave an underlying unity to her researches: in the utilization of foodstuffs; in the energy aspects of nutrition; in the nutritive values of foods as sources of protein, of calcium, and of vitamins; in the nutritional requirements for iron, with special reference to growth and development, and the nutritional availability of the iron of typical foods; in the more critically scientific determination of the nutritional characteristics of different groups of foods and the logical place of each in the diet. Planning her research as she did, with reference to the needs of our science as she saw it and without any anxiety as to individual fame, Mrs. Rose would doubtless deprecate any attempt to feature particular findings as her outstanding scientific contributions, and would say that her best discoveries are the students who have gone from her training into positions of leadership in the advancement of our science and of its functioning for human welfare.

<div align="right">H. C. S.</div>

CEREALS AND RICKETS

XIII. PHYTIC ACID, YEAST NUCLEIC ACID, SOYBEAN PHOSPHATIDES
AND INORGANIC SALTS AS SOURCES OF PHOSPHORUS
FOR BONE CALCIFICATION [1]

CARL H. KRIEGER, RUDOLF BUNKFELDT, C. RAY THOMPSON AND
HARRY STEENBOCK

*Department of Biochemistry, College of Agriculture, University of Wisconsin,
Madison*

Recent investigations in this laboratory (Krieger, Bunkfeldt and Steenbock, '40; Krieger and Steenbock, '40) revealed that in the absence of vitamin D the phosphorus of phytic acid was poorly available to the rat. In the presence of vitamin D its availability was markedly increased, although its efficiency never equalled that of inorganic phosphorus fed at the same level of intake. These findings are apparently important because approximately 40 to 70% of the phosphorus of cereal grains is present in the form of phytic acid (McCance and Widdowson, '35; Giri, '38).

In general the distribution of forms of phosphorus other than phytic acid in cereal grains has not been studied extensively. Attention, however, might be called to the following investigations. Heubner and Reeb ('08), Rising ('10) and Jebbink ('10) reported on the nucleic acid phosphorus present in grains and foodstuffs. Rengniez ('11) found that most of the phosphorus of flour was present as phytic acid and nucleic acid. Bernardini ('12) found both nucleic acid and phospholipid phosphorus present in small amounts in rice.

Stellwaag (1890), Posternak ('03), Koch ('05), Suzuki, Yoshimura and Takaishi ('07) Heubner and Reeb ('08), Vageler ('09), Rising ('10), Vorbrodt ('10) and Alpers ('19) on the other hand turned their attention to the phospholipid content of cereals.

Apparently little is known of the comparative availability of these different forms of phosphorus. Accordingly in the following experiments the phosphorus of phytic acid from wheat bran was compared in its availability with that from yeast nucleic acid and soybean phosphatides using the rat as the experimental animal.

Unless indicated otherwise the basal ration employed was the low-phosphorus ration of Schneider and Steenbock ('39). This had a percentage composition of cerelose (glucose) 49, egg white 18, cooked starch 20, a rice polish concentrate [2] 4, phosphorus-free salts 4, and cottonseed oil 5. All additions to this ration were made at the expense of the cerelose. The phosphorus additions were based on the assumption that an optimal level of phosphorus intake was secured when 100 gm. of the ration contained 0.257 gm. of phosphorus (Krieger, Bunkfeldt and Steenbock, '40). The consumption of food was equalized for all rats at any one of the various levels of phosphorus fed. The rats used weighed from 50 to 60 gm. All experiments were run for a period of 4 weeks. The technique used was the same as that employed in previous investigations (Krieger and Steenbock, '40).

Nucleic acid phosphorus versus inorganic phosphorus. The yeast nucleic acid used in these experiments was a commercial product.[3] It contained 7.50% phosphorus as determined by the Fiske and Subbarow method ('25). It was apparently free from inorganic phosphorus because none was extracted by treatment with 2% HCl for 2 hours. The inorganic phosphorus additions were made in the form of a neutral mixture of the mono and dibasic potassium phosphates. Both the

[2] Vitab.

[3] Obtained from the Pfansteihl Chemical Company, Waukegan, Illinois.

nucleic acid phosphorus and inorganic phosphorus were fed at levels of 0.032, 0.064 and 0.128 gm. per 100 gm. of ration. In addition, both nucleic acid and inorganic phosphorus were fed at a level of 0.064 with the further addition of 45 U.S.P. units of vitamin D_2 to 100 gm. of ration.

It is evident (table 1) that the phosphorus of yeast nucleic acid was as readily available as inorganic phosphorus both in the presence and in the absence of vitamin D. The addition of vitamin D improved the utilization of both.

TABLE 1

The availability of nucleic acid phosphorus

GROUP	RATION COMPOSITION (ADDITIONS TO BASAL)	NUMBER OF RATS	FOOD CONSUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	gm.	gm.	gm.	%
1	0.032 gm.[1] nucleic acid P	6	221	46	0.0998	0.0234	23.4
2	0.032 gm. inorganic P	6	208	37	0.0936	0.0213	22.8
3	0.064 gm. nucleic acid P	5	245	55	0.1153	0.0328	28.4
4	0.064 gm. inorganic P	4	241	53	0.1113	0.0315	28.3
5	0.064 gm. nucleic acid P plus vitamin D [2]	6	230	52	0.1245	0.0482	38.7
6	0.064 gm. inorganic P plus vitamin D	6	229	52	0.1263	0.0492	38.9
7	0.128 gm. nucleic acid P	6	259	70	0.1332	0.0518	38.9
8	0.128 gm. inorganic P	6	260	63	0.1345	0.0539	40.0

[1] Per 100 gm. ration.

[2] Forty-five U.S.P. units (viosterol) per 100 gm.

Further evidence of the equality of nucleic and inorganic phosphorus was obtained by inspection of the distal ends of the radii and ulnae of young rats after they had received these forms of phosphorus as a supplement to a rachitogenic diet. By way of preparation for the test, rats weighing 50 to 60 gm. were put on the low-phosphorus, rachitogenic diet of Schneider and Steenbock ('39). After 2 weeks on this diet when they were severely rachitic, they were given the supplements equivalent to 0.032 and 0.064 gm. of phosphorus in 100 gm. of ration. All the rats were given the same amount of ration.

After 5 days the amount of healing of the rachitic lesion was found to be the same for both supplements at their respective levels of intake.

Phospholipid phosphorus versus inorganic phosphorus. The phospholipid used for these experiments was a sample of re-precipitated crude soybean lecithin.[4] It was freed from most of the soybean oil carrier by extraction with acetone as follows: One hundred grams of the sample were triturated with 1000 cc. of acetone until a fine suspension was obtained. The phospholipid was then filtered off, re-extracted with 200 cc. of acetone and filtered again. The resulting waxy, light yellow product was dried in vacuo over calcium chloride in the dark. It was found to contain 2.63% of phosphorus. It was incorporated in the rations by dissolving it in ether and then evaporating the desired amount of the ether solution on the ration before a fan at room temperature. The experimental series was set up in the same manner as the preceding series with nucleic acid.

The results (table 2) show that in general phospholipid phosphorus was as available as inorganic phosphorus and that the addition of vitamin D improved the utilization of both to the same degree. However, at one level of intake, namely 0.064, inorganic phosphorus apparently produced slightly more bone ash than the phospholipid, namely 3.7%. It is possible that this difference was of no significance. However, it was deemed advisable to carry out an additional experiment. This was done by adding both sources of phosphorus to a basal ration low in both calcium and phosphorus. The calcium content of the ration was adjusted by the addition of calcium carbonate to the low phosphorus ration to give a calcium/phosphorus ratio of 1.5/1 and 10/1 respectively with the phosphorus at a level of 0.064 gm. per 100 gm. of ration.

The results of this experiment revealed slight differences in value of the phospholipid phosphorus at both calcium/phosphorus ratios. At the 1.5/1 ratio there were obtained

36.2 and 34.5% of bone ash respectively with phospholipid and inorganic phosphorus. At the 10/1 ratio the bone ashes were respectively 29.4 and 27.2%. When evaluated in relation to the slight differences in the other direction in the preceding experiments, the differences are believed to be without significance. Furthermore, inspection of the calcification of the radii and ulnae of the rachitic animals by the same technique as that employed before revealed the same amount of calcification in each.

TABLE 2

The availability of phospholipid phosphorus

GROUP	RATION COMPOSITION (ADDITIONS TO BASAL)	NUMBER OF RATS	FOOD CONSUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	*%*
1	None	6	234	34.6	0.0899	0.0197	21.8
2	0.032 gm.[1] phospholipid P	6	252	53.0	0.1042	0.0251	24.0
3	0.032 gm. inorganic P [2]	6	252	56.6	0.1059	0.0279	26.6
4	0.064 gm. phospholipid P	6	250	63.9	0.1151	0.0346	30.2
5	0.064 gm. inorganic P	6	250	73.6	0.1221	0.0412	33.9
6	0.064 gm. phospholipid P plus vitamin D[3]	6	250	73.5	0.1446	0.0626	43.3
7	0.064 gm. inorganic P plus vitamin D	6	250	65.0	0.1435	0.0612	42.8
8	0.128 gm. phospholipid P	6	267	79.9	0.1447	0.0610	42.2
9	0.128 gm. inorganic P	6	267	89.5	0.1512	0.0629	41.6

[1] Per 100 gm. ration.
[2] As KH_2PO_4 instead of neutral phosphate mixture.
[3] Forty-five U.S.P. units (viosterol) per 100 gm.

Additional data on the equivalent value of phospholipid and inorganic phosphorus were obtained in an experiment involving both growth and reproduction. In this experiment one group of rats was fed phospholipid phosphorus and another the neutral mixture of inorganic phosphorus at such a level as to incorporate 0.11 gm. of phosphorus in each 100 gm. of the low phosphorus ration. Both rations were supplemented with 100 U.S.P. units of vitamin D (viosterol) per rat per week. Rats weighing from 50 to 60 gm. were put on these diets for a period of 20 weeks. They were then bred. The average weekly gain in weight for the rats on the phospho-

lipid phosphorus diet was 13.1 gm. for the males and 10.4 gm. for the females. For the rats on the inorganic phosphates the gains were 12.9 for the males and 9.3 gm. for the females. After mating, pregnancy was readily induced and normal young were born at term. One female on the phospholipid ration failed to rear her young, but three others maintained their litters for 9 weeks. The average weekly gain in weight for the young was 10 gm. on the phospholipid ration and 9.5 gm. on the inorganic phosphate ration.

TABLE 3

The availability of nucleic acid phosphorus, phospholipid phosphorus, inorganic phosphorus and phytic acid phosphorus

GROUP	RATION COMPOSITION (ADDITIONS TO BASAL)	NUMBER OF RATS	FOOD CONSUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	*%*
1	0.064 gm.[1] nucleic acid P	6	196	44	0.1058	0.0349	33.0
2	Same plus vitamin D [2]	6	197	41	0.1283	0.0533	41.5
3	0.064 gm. phospholipid P	6	197	48	0.1003	0.0303	30.2
4	Same plus vitamin D	6	197	44	0.1182	0.0517	43.7
5	0.064 gm. inorganic P	6	197	44	0.1073	0.0349	32.5
6	Same plus vitamin D	6	197	41	0.1265	0.0523	41.3
7	0.064 gm. phytic acid P	6	194	42	0.0969	0.0229	23.6
8	Same plus vitamin D	6	197	44	0.1104	0.0411	37.2

[1] Per 100 gm. ration.
[2] Forty-five U.S.P. units (viosterol) per 100 gm.

Nucleic acid, phospholipid, phytic acid and inorganic phosphorus. The results in the preceding series, when evaluated in the relation to those obtained with phytic acid (Krieger, Bunkfeldt and Steenbock, '40; Krieger and Steenbock, '40), suggested a direct comparison of the aforementioned sources of phosphorus in one series. The nucleic acid and phospholipid preparations were the same as those used before. The phytic acid was prepared from phytin isolated from wheat bran according to the method of Boutwell ('17). Each preparation was added to the low-phosphorus basal ration in an amount equivalent to 0.064 gm. phosphorus per 100 gm. of ration. All rations were fed with and without vitamin D.

Table 3 reveals again that the phosphorus from yeast nucleic acid and soybean lecithin was the equal of inorganic phosphorus and that phytic acid was markedly inferior in this respect. Again the addition of vitamin D produced the greatest percentage improvement with phytic acid yet the resultant bone ash did not equal that obtained with the other compounds.

SUMMARY

The phosphorus of yeast nucleic acid and soybean phosphatides was found to be as readily available for calcification of bone as inorganic phosphorus. This stands in marked contrast with the availability of the phosphorus of phytic acid which is poorly available. By the addition of vitamin D the utilization of the phosphorus of phytic acid was increased in greater measure than that of the other forms of phosphorus but it was still less efficient for the production of good bone than the other sources.

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LIFE-TIME EXPERIMENTS UPON THE PROBLEM OF OPTIMAL CALCIUM INTAKE [1]

F. O. VAN DUYNE, C. S. LANFORD, E. W. TOEPFER AND H. C. SHERMAN

Department of Chemistry, Columbia University, New York

In experiments with rats, a mixture of five-sixths ground whole wheat and one-sixth dried whole milk with sodium chloride and distilled water (our diet A or laboratory diet 16) proves adequate, generation after generation, but is suboptimal in calcium and riboflavin content and in vitamin A value. In these respects it appears representative of a rather large proportion of human dietaries and we have therefore used this diet as a starting point in the experimental study of different dietary enrichments. (It is recognized, of course, that human dietaries may frequently be suboptimal in other respects also.)

EXPERIMENTAL

Our general experimental program includes enrichment of this original diet A or 16 in each of several factors (separately and in different combinations) up to the level of intake which induces its optimal response in nutritional well-being as measured by different criteria.

In previous papers it has been shown that when the calcium content is increased (without any other change in the diet than the addition of calcium salts) from the original 0.2%, to 0.35, 0.64, or 0.8% of calcium in the dry food mixture, there results in early life a relatively small increase in the rate of gain in

body weight and a relatively larger increase in the rate of gain in body calcium (Sherman and Campbell, '35, '37; Toepfer and Sherman, '36; Lanford and Sherman, '38; Sherman, Campbell and Lanford, '39); also that the 0.35% is distinctly superior to the 0.2% level throughout life (Sherman and Campbell, '35).

TABLE 1

Records of rats on diets of different calcium contents [1]

	MEANS ± THEIR PROBABLE ERRORS			
	On diet 168, 0.64% calcium		On diet 169, 0.8% calcium	
	Males	*Females*	*Males*	*Females*
Weight at 30 days, gm.	46 ± 0.4 (77)	45 ± 0.3 (105)	46 ± 0.4 (72)	45 ± 0.3 (104)
60 ,, ,,	116 ± 1 (77)	104 ± 1 (105)	119 ± 1 (72)	107 ± 1 (104)
90 ,, ,,	191 ± 2 (77)	159 ± 1 (105)	193 ± 2 (72)	160 ± 1 (104)
180 ,, ,,	303 ± 2 (77)	198 ± 1 (104)	305 ± 2 (72)	203 ± 1 (103)
365 ,, ,,	349 ± 3 (75)	231 ± 1 (99)	349 ± 3 (71)	237 ± 1 (101)
Age at birth of first young, days		111 ± 1 (102)		110 ± 1 (103)
Duration of reproductive life, days		311 ± 10 (105)		360 ± 10 (104)
Young born per female		38.6 ± 1.3 (105)		41.9 ± 1.35 (104)
Young reared per female		24.7 ± 0.9 (105)		27.0 ± 1.0 (104)
Avg. wt. of young at 28 days, gm.	40.2 ± 0.1 (2589)		40.1 ± 0.1 (2805)	
Length of life, days	734 ± 10 (77)	777 ± 13 (105)	729 ± 11 (71)	824 ± 11 (104)

[1] Number of cases given in parentheses under each mean.

The purpose of the present paper is to record the results of life-time and successive-generation experiments at the higher levels of 0.64 and 0.8% of calcium in the dry food; and to consider the question whether at these levels a definite "plateau of optimal nutritional response" (for such condi-

tions as these experiments represent) has now been located. The data here reported include experiments with 148 males and 209 females observed until natural death.

Table 1 compares the effects of the two diets here reported (diet 168 with 0.64%, and diet 169 with 0.8% calcium) upon body weight at different ages, duration of reproductive life, success of the females in bearing and rearing young, and length of life of each sex.

DISCUSSION

The weights at all ages from 1 month to 1 year, the ages of the females at the birth of their first young, the average weight of the offspring at 28 days of age, and the longevity of the males are substantially alike for these two diets. The females on the diet of higher calcium content, however, retained reproductive ability for a longer time, bore more young, reared more young, and lived longer. Any one of these differences taken alone and viewed in the light of its probable error may seem to be of doubtful statistical significance; but when all the observed differences fall to the credit of the same diet, it may be regarded as a matter for individual judgment whether or not the evidence shows a slight superiority in diet 169 over diet 168. As there were over 100 females on each of these diets, the effects of individual variation were presumably as well minimized as is practicable in long-time controlled experimentation.

Hence for such conditions as are here described, it appears that 0.64% of calcium in the dry food is either on or just at the beginning of the "plateau" of optimal intake for permanent nutritional well-being; and that 0.8% of calcium is certainly within this optimal region. How much above 0.8% this optimal zone extends, i.e., at what level a further increase of calcium intake would begin to be unfavorable in its effect upon permanent nutritional well-being, is a question not included in our problem.

CONCLUSION

In a dietary adequate in all respects but suboptimal in riboflavin content and vitamin A value a calcium intake of 0.64 to 0.8% of the dry food, or about three to four times that of minimal adequacy, gave best results in permanent nutritional well-being, as shown by full-life experiments in three generations of rats maintained under laboratory conditions.

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UNCOMPLICATED VITAMIN E DEFICIENCY IN THE RABBIT AND ITS RELATION TO THE TOXICITY OF COD LIVER OIL

C. G. MACKENZIE, JULIA B. MACKENZIE AND E. V. McCOLLUM ·
Department of Biochemistry, School of Hygiene and Public Health,
The Johns Hopkins University, Baltimore, Maryland

In 1931 Goettsch and Pappenheimer ('31) reported the production of extensive lesions of the voluntary muscles of rabbits and guinea pigs fed a natural food diet treated with ferric chloride, and supplemented with lard and cod liver oil (diet 13). Soon thereafter there appeared the first of a series of papers by the Cornell workers, later incorporated in a bulletin (Madsen, McCay and Maynard, '35), on the toxicity of cod liver oil for rabbits, guinea pigs, goats and sheep. These workers found that the addition of cod liver oil to synthetic and natural food diets produced muscle lesions, and though the dystrophic action of the synthetic diet (containing lard) was delayed when cod liver oil was omitted it was not entirely abolished. They concluded that both cod liver oil and some other factor of the synthetic diet were causative agents. Madsen ('36) noted the beneficial effect of cottonseed oil in dystrophy-producing diets. Davis, Maynard and McCay ('38) confirmed this observation, but found that cottonseed oil did not completely protect guinea pigs against dystrophy when cod liver oil concentrates were administered. They state that while it appears that a specific injurious factor is present in the oil, such an explanation must be considered tentative only.

We have shown (Mackenzie and McCollum, '39, '40) that muscular dystrophy in rabbits maintained on diet 13 plus

defatted wheat germ can be prevented or cured by alpha-tocopherol. Furthermore, we have been unable to confirm the report of Morgulis ('38) that a water-soluble factor is also required for maintenance of the integrity of the rabbit's skeletal muscles (Mackenzie, Levine and McCollum, '40). Morris ('39) found that rabbits rendered dystrophic on an alfalfa and grain diet through the oral administration of cod liver oil were cured by alpha-tocopherol. Shimotori, Emerson and Evans ('39, '40) were able to prevent dystrophy in guinea pigs fed a synthetic diet containing lard and cod liver oil through the administration of alpha-tocopherol. The California workers administered the cod liver oil and the alpha-tocopherol on alternate days. These results suggested that vitamin E, when properly administered, prevented the harmful action of cod liver oil on the voluntary muscles of the rabbit and guinea pig.

The next question in our investigations concerning the biological action of vitamin E was its mode of action in preventing the severe muscle lesions in the rabbit. Is it in itself an essential factor (readily inactivated by cod liver oil) in the metabolism of these animals, or does it protect them in some way from the toxic action of animal fats to which they are particularly sensitive? The delayed appearance of symptoms when cod liver oil was omitted from the synthetic diet (containing lard) of Madsen et al., the absence of dystrophy in goats and rabbits fed a vitamin E-deficient diet for more than a year by Thomas and Cannon (cited by Mattill, '38), and the months required to develop the neuro-muscular symptoms in rats reared on the most highly purified vitamin E-deficient diet yet used (Mackenzie, Mackenzie and McCollum, '40) suggested the latter explanation.

On the other hand, the observation on rats of Weber et al. ('39) that rancid fats destroy vitamin E when the two are mixed together in the diet, and that this destruction does not occur when rancid fats and vitamin E are fed separately 6 hours apart (the result of mixing in the gastrointestinal tract without previous contact in the diet was not studied) favored the first hypothesis. Mattill ('40) has reported that the vita-

min E content of a synthetic diet containing lard and cod liver oil gradually declines on standing, and that the loss of vitamin E may be delayed by the use of appropriate stabilizers against oxidative rancidity. He (Mattill, '38) has suggested that autoxidative rancidity of animal fats included in dystrophy-producing diets may be the principal cause of the disorder; vitamin E is antioxygenic in these fats and therefore it disappears in their presence even before rancidity becomes evident. Herbivorous animals have a large cecum where the food remains long enough for autoxidative changes to progress farther and more rapidly than in omnivorous animals. For a

TABLE 1

DIET	E 2	E 3
Casein (defatted)	150	150
Dextrin (cornstarch)	610	690
Lard (fresh)	80	
Yeast [1]	100	100
Salt mixture [2]	60	60
Carotene	0.05	0.1
Calciferol [3]	0.0004	0.0008

[1] Northwestern yeast foam tablet powder.
[2] Salt mixture No. 51 + Mn (Mackenzie, Mackenzie and McCollum, '39).
[3] Kindly supplied by Dr. C. E. Bills of Mead Johnson & Company.

discussion of the relationship between vitamin E, auto-oxidizing fats and antioxidants, the reader is referred to the review of Mattill ('38). So far as we know the destruction of vitamin E in the gastrointestinal tract or in the body by ingested cod liver oil has not been experimentally demonstrated.

The experiment reported in this paper was designed to test the effect on the rabbit of a vitamin E-deficient diet containing but traces of animal fat.

METHODS

The composition of the two basal diets used is given in table 1. Lard was included in diet E 2 as a source of the essential fatty acids and to determine the dystrophic effect of fresh lard. The casein was prepared by washing with acetic acid and

extracting in a Lloyd extractor with cold 70% and cold 95% alcohol for 24 hours each. It was the only source of animal fat in diet E 3 and according to the method of Shaw ('20), an adaptation of the Roese-Gottlieb procedure, contained but 0.25% of lipids.

The dextrin was ground in a Hobart burr mill with the burrs set at the number 5 position and was freed of the fine particles by sifting on a 20-mesh sieve. The carotene and calciferol were taken up in ethyl ether and evaporated on the casein and dextrin. The diets were mixed thoroughly and 125 cc. of tap water per kilogram was added to bind the components together. The rations were freshly prepared once or twice a week and were stored in the refrigerator. Roughage was not included in these diets because we wished to keep them as purified as possible. Previous experience with the components of both diets had shown that they were deficient in vitamin E.

Supplements. A solution of synthetic alpha-tocopherol [1] in ethyl laurate, 20 mg. per cubic centimeter, was employed as the source of vitamin E. The cod liver oil used was of a good medicinal grade. Both the tocopherol solution and the cod liver oil were kept in the refrigerator in glass stoppered flasks. The alpha-tocopherol was always administered by mouth from a 1 cc. tuberculin syringe equipped with a long blunt needle. The cod liver oil was either given in the same way or mixed in the diet daily.

Care of animals and diagnosis of dystrophy. The animals were housed in individual cages equipped with raised screen floors. They were weighed, given fresh diet, and their food consumption determined each day. At this time diet E 3 was again moistened with tap water. Contrary to our previous procedure the rabbits were allowed continuous access to the diets.

In these experiments we relied mainly on weight, food consumption and behavior for detecting the onset of dystrophy. Sections of voluntary muscles were examined microscopically in all cases at the end of the experiment. The muscle blocks

[1] We are indebted to Merck & Company, Inc., for the supply of alpha-tocopherol.

were fixed in Zenker-formol, imbedded in paraffin and stained with hematoxylin and eosin.

Low fat and lard diets. Young rabbits weighing from 230 to 600 gm. were placed on diet 13 of Goettsch and Pappenheimer, from which the lard, cod liver oil and ferric chloride had been omitted. Beginning with the fourth day the purified diets were gradually added and by the eighth day the transfer to these diets was complete. At the end of this period, which is included in the experimental data, the controls were started on alpha-tocopherol at a level of 3 mg. a day, 6 days a week. Since the behavior of animals on diets E 2 and E 3 was similar, the results obtained on the two diets will be reported together.

The growth of rabbits on the purified diets supplemented with alpha-tocopherol averaged from 85 to 100 gm. a week. Although a loss in weight for 2 or 3 days frequently occurred, the weekly weights showed a consistent gain. Throughout the experiment some of the rabbits had watery feces intermittently for periods of several days. This seldom occurred in animals whose initial weight was less than 400 gm. Diarrhea was observed in only a few cases of severe dystrophy. The average daily food intake was 30 gm. during the first 3 weeks of the experiment and 35–43 gm. thereafter. While these diets were not optimal for growth in the rabbit, they proved quite suitable for our purpose. Lard was apparently not an essential component of the diet during the duration of the experiment.

The vitamin E-deficient animals grew as well as their controls for 3 weeks. They then either became very sick and lost weight rapidly or continued to grow, but at a reduced rate, throughout the remainder of the experiment. The average daily food intake of the surviving animals was 25 gm.

Gross symptoms of dystrophy (Mackenzie and McCollum, '40) appeared in all of the vitamin E-deficient rabbits in 2 to 4 weeks. Unlike the results obtained on diet 13 this was not always an indication of impending death. Except for the

muscular weakness, many of the animals appeared healthy 5 to 7 weeks later when they were sacrificed.

Microscopic examination of the thigh muscles and diaphragms from E-deficient animals killed at the twenty-third to seventieth day of the experiment invariably revealed severe degeneration. The muscles of the controls were normal. The

TABLE 2

The production of nutritional muscular dystrophy in young rabbits on a low fat diet (E 3), and on a low fat diet plus lard (E 2) and its prevention with alpha-tocopherol

RABBIT NO.	SEX	DIET	ALPHA-TOCOPHEROL[1]	INITIAL WEIGHT	MAXIMUM WEIGHT	TIME OF MAXI-MUM WEIGHT	FINAL WEIGHT	DURATION OF EXPERIMENT	FIRST GROSS SYMPTOMS	MICROSCOPIC LESIONS	
										Thigh muscles[2]	Diaphragm
			mg.	*gm.*	*gm.*	*days*	*gm.*	*days*	*days*		
89	♀	E 3		550	930	70	930	70	30	+ +	+ +
91	♂	E 3	3	600	1510	100	1510	100		—	—
90	♂	E 3		590	950	70	950	70	21	+ + + + + + + +	+ +
92	♂	E 3	3	550	1560	100	1560	100		—	—
129	♂	E 3		360	580	32	520	37	17	+ + + +	+ + +
128	♀	E 3	3	370	920	37	920	37		—	—
132	♂	E 3		320	680	37	680	37	29	+ + + +	+ + +
133	♀	E 3	3	240	740	37	740	37		—	—
85	♀	E 2		410	780	21	620	23	22	+ + ±	+ + + +
86	♀	E 2	3	410	1320	55	1320	55		—	—
87	♀	E 2		340	640	26	510	29	16	+ +	+ +
88	♀	E 2	3	290	1800	105	1800	105		—	—
94	♀	E 2		540	1180	70	1180	70	28	+ + + + +	+ + +
93	♀	E 2	3	580	1750	77	1700	100		—	—
95	♂	E 2		530	750	27	630	32	24	+ +	+ +
96	♂	E 2	3	490	1550	84	1500	100		—	—

[1] Alpha-tocopherol given 6 days a week.   [2] Biceps femoris and vastus lateralis.

results obtained on a group of sixteen animals are summarized in table 2. It is apparent that the degree of muscle damage is not correlated with loss of weight or the duration of gross symptoms.

The lesions generally equaled in severity and extent those observed by us in a large series of rabbits rendered dystrophic

on diet 13. They were characterized by hyalinization and necrosis of the muscle fibers and by large accumulations of cellular elements, and were undistinguishable from those produced on diet 13. In several cases, however, the changes surpassed any that we had previously seen. The greater part of the muscle had been replaced by fibrous connective tissue containing an occasional intact muscle fiber with well-preserved cross striation. Scattered through the sections were hyalinized fibers and aggregates of cellular elements showing that degeneration was still in progress. The extensive disappearance of muscle fibers, with their replacement by connective tissue, was undoubtedly a consequence of the prolonged life of severely dystrophic animals on the purified diet as contrasted with diet 13. It was certainly not due to prolonged chronic dystrophy (Mackenzie, Levine and McCollum, '40).

The effect of cod liver oil on the low fat diet supplemented with alpha-tocopherol. Young rabbits were fed diet E 3 plus 3 mg. of alpha-tocopherol daily by mouth. Either 2 cc. of cod liver oil was mixed in the diet daily or 1 cc. was administered orally soon after the daily alpha-tocopherol supplement. The results were the same in both cases.

During the 5 weeks that the animals were kept on this regimen growth and food consumption paralleled that observed on the low fat vitamin E-deficient diet. Physical symptoms of dystrophy appeared at the fourth week. The daily creatine excretion on the thirty-fourth and thirty-fifth days was from 70 to 80 mg. per kilogram of body weight. Microscopic examination of voluntary muscles taken when the animals were killed at 5 weeks revealed lesions at least as severe as those produced on diet E 3 in the same length of time. These lesions, scored as 4 or 5 plus, were similar to those produced on diet E 3 without alpha-tocopherol or cod liver oil. It should be noted here that the consumption of 2.9 gm. of lard daily (diet E 2) for 14 weeks produced no lesions in rabbits receiving alpha-tocopherol at the 3 mg. level.

DISCUSSION

Young rabbits fed a vitamin E deficient diet containing 0.05% of animal fat develop severe lesions of the voluntary muscles. These lesions are prevented by synthetic alpha-tocopherol. It may be concluded, therefore, that the ingestion of animal fats (cod liver oil in particular) is not a prerequisite for the production of nutritional muscular dystrophy in rabbits, and that vitamin E is essential for the normal structure of their voluntary muscles.

The preventive action of alpha-tocopherol, administered orally, was rendered ineffective by the oral administration of cod liver oil a few minutes later or its addition to the basal diet. Muscle lesions were produced resembling those observed on the low fat, vitamin E-deficient diet. Fresh lard added to the diet did not possess this dystrophic action.

The dystrophic action of cod liver oil fed separately from the vitamin E may be explained in several ways: (1) The destruction of alpha-tocopherol by cod liver oil following the ingestion of these two substances. (2) A toxic action of cod liver oil, unrelated to vitamin E, but producing similar morphological changes in the voluntary muscles. (3) A destructive action of cod liver oil on the muscles that is prevented by vitamin E either by virtue of its vitamin activity, or its activity as an antioxidant (Olcott and Emerson, '37) if indeed these two functions are independent. In this case the vitamin requirement should parallel the cod liver oil intake.

The observation of Shimotori et al. ('40) that guinea pigs on a purified diet plus cod liver oil are protected against dystrophy by alpha-tocopherol (the alpha-tocopherol and cod liver oil being administered on alternate days), and the experiments reported in this paper in which the action of alpha-tocopherol was rendered ineffective by cod liver oil when no precautions were taken to prevent the mixing of the vitamin and the oil in the gastrointestinal tract make the first explanation the most likely. If this should prove to be the case it would probably also account for the action of cod liver oil on the voluntary muscles of other animals. However, additional work is

needed for the establishment of this hypothesis as the correct one. This is particularly so in view of the report of Davis, Maynard and McCay ('38) on the dystrophic action of cod liver oil concentrates. It must be borne in mind that the possible explanations offered above do not necessarily exclude one another.

SUMMARY

1. Vitamin E deficiency in rabbits maintained on a purified low fat diet results in the rapid development of severe lesions of the voluntary muscles. These lesions are prevented by synthetic alpha-tocopherol.

2. The preventive action of alpha-tocopherol is rendered ineffective when the vitamin and cod liver oil are administered orally within a few minutes of each other at the levels employed, or when the oil is added to the basal diet.

The histological preparations used in this experiment were made by Miss Miriam Reed.

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RELATION OF PARATHYROID FUNCTION AND DIET TO THE MINERAL COMPOSITION OF THE BONES IN RATS AT THE CONCLUSION OF PREGNANCY [1]

M. BODANSKY AND VIRGINIA B. DUFF

The University of Texas Medical School, and the
John Sealy Memorial Research Laboratory, Galveston

ONE FIGURE

The complications developing toward the end of pregnancy in parathyroid-deficient rats appear to be related to the marked depression of the serum calcium. A diet may be adequate for the normal pregnant rat and fairly so for the parathyroidectomized non-pregnant rat, yet fail to maintain the calcium concentration of the blood of the parathyroid-deficient pregnant rat at anywhere near the normal level. From this it may be deduced that regulation of the blood calcium in the pregnant organism depends, even more than in the non-pregnant animal, on its ability to mobilize calcium from its reserves or from a high calcium intake. In order to show how parathyroid deficiency affects the calcium and phosphorus economy of pregnant animals, the mineral composition of the bones of parathyroidectomized rats following one or more pregnancies was compared with that in unoperated control rats.

It is common knowledge that demineralization of bone occurs during pregnancy, especially if the mineral composition of the diet falls short of the requirements. It is also recognized that

such changes as may accompany a single pregnancy are augmented by repeated pregnancies, and are, therefore, more readily demonstrable. This is illustrated by the data in table 1 which permit comparisons between parathyroidectomized rats and their normal pregnant controls: (1) on diet no. 7 (Ca 0.49%, P 0.49%), two pregnancies; (2) on diet no. 7, three pregnancies; (3) on diet no. 27 (Ca 0.122%, P 0.245%), three pregnancies. The experimental procedure and diets are described in a previous paper ('41).

The data in table 1 bring out several significant points which are more amply confirmed by the data represented in figure 1 and by those in table 2. The tabulated data show that in our experiments the parathyroid-deficient animals differed from the corresponding controls in two important respects. The ash content of the bones (humeri and femurs, dried and defatted) tended to be higher in the parathyroid-deficient animals than in the controls. Even more conspicuous (tables 1 and 2; fig. 1) were the heavier weights, both absolute and relative to the pregestational body weight, of the bones (two humeri and two femurs) in the parathyroid-deficient rats. At this point it is necessary to explain that the last pregestational weight was taken as a more reliable basis for calculation than the weight at any stage of pregnancy or that following parturition. This convention eliminated errors due to different rates of gain in weight during pregnancy in normal and parathyroidectomized rats, variable loss of weight toward the end of gestation in parathyroidectomized and rarely in normal rats because of loss of appetite during the last 2 or 3 days, differences in size and weight of litters, and other factors.

From the data in table 1, it is seen that the coefficient, bone weight/body weight × 1000, and the ash content of the bones were invariably higher for the parathyroprevic rats than for the corresponding controls.

The data for rats 324 and 354 are included for the purpose of recording the lowest bone weight/body weight coefficients encountered in the present series of experiments. As may be anticipated, these occurred in unoperated control rats maintained for long periods on rations deficient in calcium. One

had been fed diet no. 27 (Ca 0.122%, P 0.245%) for a period of more than 7 months, during which time it gave birth to four litters; the other had been fed diet no. 26 (Ca 0.017%, P 0.245%) for a shorter period (104 days), during which time it gave birth to three litters. The ash content and the per cent of calcium of the bones were significantly lower than in the unoperated pregnant rats fed diet no. 7.

The curves in figure 1 are based on the results obtained in normal and parathyroid-deficient pregnant rats maintained

TABLE 1

Influence of repeated pregnancies on composition of bones in parathyroid-deficient (P-D) rats and their normal controls (C)

Rat no.	529	458	304	447	364	78	354	324
	C	P-D	C	P-D	C	P-D	C	C
Diet no.	7	7	7	7	27	27	26	27
Duration of diet, months	2	3⅗	4½	8½	4	5	3¼	7¼
Number of pregnancies	2	2	3	3	3	3	3	4
Per cent of ash[1]	64.75	66.56	66.00	69.09	62.10	66.60	54.75	58.03
Calcium, per cent of ash[1]	36.80	36.77	36.18	36.55	36.32	38.66	34.32	35.84
Phosphorus, per cent of ash[1]	18.46	18.68	17.65	16.23	17.96	17.88	17.50	17.12
Calcium-phosphorus ratio	1.99	1.97	2.05	2.25	2.02	2.16	1.96	2.09
Weight of bones[1] (2 humeri, 2 femurs) in gm.	1.094	1.302	1.154	1.387	1.045	1.345	0.728	0.867
Last pregestational body weight in gm.	198	198	240	221	196	217	205	224
$\frac{\text{Bone weight}}{\text{Rat weight}} \times 1000$	5.53	6.57	4.81	6.27	5.33	6.20	3.55	3.87

[1] Footnote to table 2 applies also to the data in this table.

on diet no. 7, the calcium and phosphorus content of which is such as to assure maximum reproductive success in normal rats (Cox and Imboden, '36; Bodansky and Duff, '41). The lower curves represent the observations in animals that had passed through one successful reproductive cycle, while the upper curves represent observations in animals that had gone through two such cycles. Admitting that the numbers of observations in each of these subgroups were small and that the bone weight/body weight coefficients showed a wide range of

individual variation, the results reveal, nevertheless, the important effect of intact parathyroid function on the mineral reserves of bone during pregnancy. Thus, at the conclusion of one pregnancy the mode for the bone weight/body weight coefficients of fifteen rats with intact parathyroids was approximately 5.3, while that for thirty-nine parathyroidectomized rats was approximately 6.0. A second pregnancy caused an even further reduction in the mean and modal values for the bone weight/body weight coefficients of the normal rats.
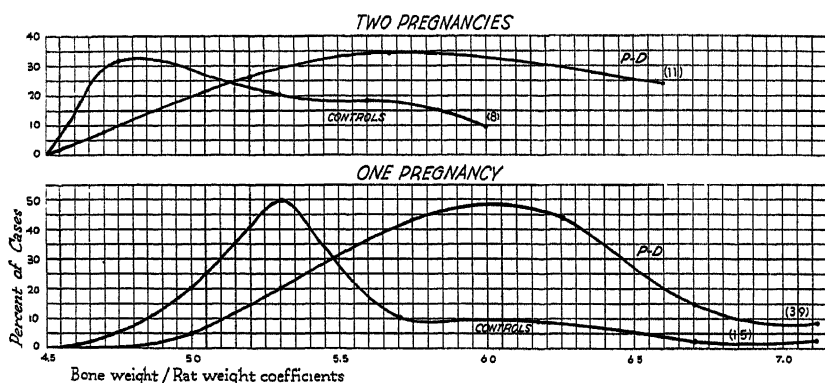
Fig. 1   Curves showing the effect of presence or absence of parathyroids on the weight of the bones (femura and humeri; dried, alcohol and ether extracted) in rats at the conclusion of one and two pregnancies. Abscissas represent the ratios: bone weight/pregestational body weight × 1000. Ordinates represent the per cent of rats in each category. Curves for the parathyroid-deficient rats are designated by the letters P–D. The figures in parentheses denote the number of rats in each group.

In table 2 are summarized the data obtained on the various diets. It will be seen that, excepting diet no. 16, the bone weight/body weight ratios were invariably lower in the controls than in the parathyroid-deficient rats. The long bones of the latter, on the different diets, averaged 10 to 20% heavier than those of the controls. There was, moreover, a higher ash content in the bones of the parathyroid-deficient animals. In contrast to these findings, the average bone weight coefficient and the average per cent of ash were of approximately the same magnitude for both the operated and unoperated rats fed diet no. 16 (Ca 1.225%, P 0.245%, Ca/P

TABLE 2

Influence of pregnancy on composition of bones in parathyroid-deficient (P-D) rats and their normal controls (C)

DIET NO.		NUMBER OF ANALYSES	PER CENT ASH	COMPOSITION OF ASH [1]		AVERAGE Ca/P RATIOS	BONE WEIGHT × 1000 / RAT WEIGHT
				Per cent Ca	Per cent P		
7	C	28	65.25 ± 2.21	36.82 ± 1.27	18.32 ± 0.67	2.01	5.426 ± 0.669
	P-D	45	66.62 ± 2.23	36.31 ± 1.54	18.23 ± 0.81	1.99	6.023 ± 0.573
8	C	4	65.12 ± 2.62	36.56 ± 0.76	18.16 ± 1.22	2.01	4.712 ± 0.339
	P-D	2	67.22 ± 0.04	36.57 ± 0.61	17.94 ± 0.46	2.04	5.503 ± 0.038
10	C	5	65.20 ± 1.83	37.04 ± 1.82	17.92 ± 1.02	2.07	5.052 ± 0.558
	P-D	1	67.50	36.78	17.46	2.11	6.305
12	C	6	66.12 ± 2.21	36.86 ± 0.93	18.01 ± 1.08	2.05	5.146 ± 0.489
	P-D	7	66.93 ± 2.60	37.09 ± 0.94	18.21 ± 0.92	2.04	5.833 ± 0.356
16	C	7	63.99 ± 1.91	37.11 ± 0.45	18.58 ± 0.83	2.00	5.360 ± 0.232
	P-D	8	64.25 ± 3.34	36.95 ± 0.31	18.50 ± 0.99	2.00	5.264 ± 0.394
26	C	15	63.53 ± 3.50	37.60 ± 0.72	18.26 ± 1.24	2.06	4.738 ± 0.522
	P-D	9	65.69 ± 2.15	36.52 ± 0.48	18.88 ± 0.48	1.93	6.032 ± 0.362
27	C	11	63.41 ± 1.88	37.24 ± 1.41	18.02 ± 0.58	2.07	5.297 ± 0.347
	P-D	13	64.98 ± 2.47	37.25 ± 1.55	18.17 ± 0.31	2.05	6.229 ± 0.362
19	C	5	66.55 ± 1.57	36.75 ± 0.43	18.75 ± 0.41	1.96	5.609 ± 0.411
	P-D	4	68.86 ± 1.35	36.04 ± 0.39	18.72 ± 0.37	1.93	6.312 ± 0.402

[1] Calcium was determined by titration of the oxalate with permanganate, following the technique of A. T. Shohl and F. G. Pedley (1922, J. Biol. Chem., vol. 50, p. 537). Phosphorus was determined by adapting the method of A. T. Shohl and H. B. Brown, as described by Van Slyke and Peters in Quantitative Clinical Chemistry, vol. 2, p. 869.

Preliminary to analysis, the bones were freed from as much tissue as possible, dried at 105°C. to constant weight, scraped carefully, without loss of bone substance, broken into several pieces and extracted for 18 hours with boiling alcohol and then for 18 hours with boiling ether. The bone weights, as well as the analytical data, represent the alcohol- and ether-extracted, dried bones.

ratio 5.0). The explanation presumably lies in the fact that this diet is rachitogenic and that its effects in promoting mobilization of bone salts and in retarding bone growth more than offset the effects of parathyroid deficiency. On diet no. 16 (Bodansky and Duff, '41), the pregnant rats did not exhibit the usual symptoms of parathyroid deficiency, the serum calcium being maintained at a high level throughout pregnancy.

DISCUSSION OF RESULTS

In interpreting the bone changes, consideration must be given to the fact that the rats used in our experiments had not attained full growth. Hammett ('23, '24) has shown that anatomical and chemical differentiation of bone is significantly altered in the growing female rat in the absence of parathyroid secretion. He studied particularly the changes produced during the 50-day interval between 100 and 150 days of age. In females, as in males, lack of parathyroid function caused moderate retardation of absolute growth in weight and length of the humerus and femur. No distortion of anatomical or chemical differentiation of these bones was apparent in the male rats, but in the females, the long bones were not only retarded in growth, but contained a lower per cent of ash, a lower per cent of calcium, and somewhat higher percentages of phosphorus and magnesium than in normal animals.

These changes, characteristic of the parathyroid-deficient, growing rat, were more than offset in our experiments by the effects of pregnancy. The mechanisms which come into play during pregnancy tend to produce in rats with intact parathyroid function demineralization and retarded bone growth to such a degree that the bones (femurs and humeri) weigh less and have a lower mineral content than the corresponding bones of the parathyroid-deficient pregnant rats.

The significance of these findings is apparent. In response to pregnancy the normal rat draws, to a considerable degree, on the calcium and phosphorus reserves of the bones and perhaps on other sources, while in rats without parathyroids, the usual mechanism for the mobilization of bone salts is abolished. The failure of this mechanism is clearly brought out by the

close similarity of the bone weight coefficients for the parathyroidectomized pregnant rats fed rations as diverse in calcium content as 0.49% (diet no. 7), 0.122% (diet no. 27), and 0.017% (diet no. 26). On the other hand, in rats with intact parathyroids significant differences occurred on these diets. As shown in table 2, the average bone weight coefficient was somewhat higher for the control rats fed diet no. 7 than for those fed diet no. 27 and distinctly higher than for those fed diet no. 26.

From these considerations it does not follow that the contrasting bone changes in the parathyroidectomized and control rats were necessarily due to abnormal excretion of calcium in the latter group. Moderate degrees of decalcification may in fact accompany positive calcium and phosphorus balances, indicating a possible relationship to the redistribution of minerals incidental to continued body growth. The normal pregnant rat frequently shows a considerable absolute gain in weight during pregnancy, whereas the parathyroid-deficient rat, despite equivalent food consumption, usually shows little or no change.

The average calcium-phosphorus ratios of the ash fell within a narrow range of variation, were apparently unaffected by the composition of the diet, and did not appear to be significantly different in the control rats than in the parathyroid-deficient rats (table 2). However, occasionally, high values were encountered in individual cases (for example, rats 78 and 447, table 1).

In planning our experiments, the main objective was to determine the effects of parathyroid deficiency in the pregnant rat. Unfortunately, we did not anticipate the possible value of data obtained in virgin rats fed the same diets, although analyses were made of the bones of rats which failed to become pregnant. On the basis of the data thus obtained, certain statements may be made tentatively. In rats with intact parathyroids the bone weight coefficients tended to be somewhat higher for the non-pregnant rats than for the pregnant rats, the greatest discrepancy occurring when the calcium intake was very deficient (diet no. 26). This relation seemed

to be reversed in the parathyroidectomized rats. Thus, the average coefficient for eight sterile rats on diet no. 7 was 5.77, compared to 6.023 for the pregnant group (table 2). The average for nine sterile rats on diet no. 26 was 5.71, compared to 6.032 for the pregnant group. The full significance of these differences is obscure, though they may be related partly to differences in food consumption and rates of growth.

SUMMARY

Failure to maintain the serum calcium at a normal level in the pregnant, parathyroidectomized rat suggests a deficiency in the mobilization of calcium from bone. This deficiency in mobilization of the bone salts is also demonstrated by the finding that in general the long bones obtained at the conclusion of a pregnancy weighed more, in relation to the pre-gestational body weight, in the parathyroid-deficient rats than in the controls. The per cent of ash of the bones was likewise higher in the parathyroid-deficient rats than in the controls, while the calcium-phosphorus ratios were, on the average, nearly identical.

These relations held for various levels of calcium and phosphorus intake, the exception being diet no. 16 of Cox and Imboden. On this diet, which is rachitogenic because of its high calcium content and high calcium-phosphorus ratio, the disparity in the bone weight coefficients between normal and parathyroidectomized pregnant rats was abolished.

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A DEFICIENCY DISEASE OF FOXES PRODUCED BY FEEDING FISH

B₁ AVITAMINOSIS ANALOGOUS TO WERNICKE'S DISEASE OF MAN [1]

R. G. GREEN,[2] W. E. CARLSON [3] AND C. A. EVANS [4]

Department of Bacteriology and Immunology, University of Minnesota,
Minneapolis, Fromm Laboratories, Grafton, Wisconsin, and Fish and
Wildlife Service, United States Department of the Interior

Chastek paralysis, a dietary disease in which the principal clinical and pathologic characteristics are referable to the central nervous system, has become a major disease problem of the fox-raising industry. Clinically, the syndrome is characterized by anorexia, weakness, progressive ataxia and spastic paraplegia, with death occurring within 48 to 72 hours after the onset of neurologic symptoms. Hyperesthesia is common and the foxes seem to be in great pain, as they moan continuously in the advanced stages of the disease. At autopsy the liver usually shows severe degeneration and in the brain diagnostic vascular lesions identical with those of Wernicke's disease of man are found bilaterally and are located principally in the paraventricular grey matter.

A brief description of this disease and of its relation to Wernicke's hemorrhagic polioencephalitis has been published (Green and Evans, '40) and a report of pathologic studies is now being prepared for publication. A general description of several outbreaks of this disease and a consideration of the nutritional factors involved are the subjects of this paper.

[1] This study was carried on with the aid of the Minnesota Department of Conservation.
[2] Department of Bacteriology, University of Minnesota, Minneapolis.
[3] Fromm Laboratories, Inc., Grafton, Wis.
[4] Fish and Wildlife Service, U. S. Department of the Interior.

Rations employed on fox farms vary considerably but are usually made up of a thoroughly ground mixture of either horse or rabbit meat, ground cereal, and a variable proportion of vegetables such as carrots and tomatoes. Fish is not generally used, but an occasional fox farmer has added it to the ration as an economical substitute for part of the meat component.

Careful study of the dietary records on ranches where Chastek paralysis occurred convinced us several years ago that the cause of this disease was the feeding of fresh fish (usually frozen) as 10% or more or the ration. From pathologic studies, as well as from dietary records on ranches where outbreaks were observed, we were led to the further conclusion that the foxes were actually dying of a vitamin-B_1 deficiency. Although these two latter statements appeared to be so unrelated as to be almost inconsistent, data supporting each were sufficiently strong to warrant experimental study. A recently completed experiment confirms both conclusions in their entirety.

In 1932 and in 1936, groups of foxes totaling about 300 animals were fed diets containing fish. Chastek paralysis was produced in some groups and not in others. This year we have learned that the disease may be consistently produced by a ration containing fish if the foxes are placed in pens with elevated, screened floors, so that their feces fall to the ground and no grass is available to them.

In an experiment begun on July 12, 1940, and terminated on August 25, forty-six foxes were used. All animals were kept in pens with elevated, screen floors throughout the experiment. A diet of the sort which is known to be entirely satisfactory for the raising of foxes was made up and to this was added frozen carp in the amount of 20% of the ration.

The basic diet in this experiment had the following percentage composition: cereal 15, bread 10, carrots 9, horse meat 45, carp 20, and cod liver oil 0.5. The cereal was a mixture of ground wheat, corn, oats and rice. The meat was all horse meat from frozen supplies except on 2 days when recently slaughtered animals were used. The carp was used whole—

heads, scales and viscera being included. All ingredients of the diet were ground together to a mixture approximating the consistency of hamburger.

The foxes used had been on the regular ranch diet prior to the time the experiment was begun. On July 12 these animals were divided into three groups and given diets as follows: One group of eighteen foxes received the basal diet plus 20 mg. of nicotinic acid for each fox daily. In a second group of sixteen, each animal was fed the basal diet plus 25 mg. of thiamine hydrochloride daily. Nothing was added to the basal diet for a third group of twelve foxes.

All foxes receiving the basal diet alone and all those being fed supplements of nicotinic acid went off feed on July 24, 12 days after the experiment was begun and remained off feed until it was ended. Only those foxes receiving the basal diet plus thiamine hydrochloride maintained normal appetites and remained symptom-free and in good health throughout the experiment.

Fatalities began to occur in the control group receiving no supplement of vitamins on August 1, 20 days after the experiment began. Seven of the twelve foxes in this group were dead of typical Chastek paralysis by August 15, a mortality of more than 50%.

In the group fed the basal diet plus nicotinic acid, two foxes died on July 30, two more died on August 1, and eleven of the eighteen foxes had died of Chastek paralysis by August 18.

Foxes in the third group were fed exclusively on the basal ration plus thiamine until August 25 and remained entirely normal throughout the experiment.

Results of this experiment show firstly that Chastek paralysis may be produced at will by feeding a diet containing 20% fresh carp. In no case have we observed an outbreak of Chastek paralysis on a ranch where fish or fish products constituted less than 10% of the total ration and, since most fox ranchers do not feed fish to their animals, this fact alone provides strong evidence that Chastek paralysis is caused by the

feeding of fish. All available experimental evidence further supports this conclusion.

Because none of the sixteen foxes receiving the basal diet plus the supplement of thiamine hydrochloride showed any evidence of illness and inasmuch as Chastek paralysis was allowed to progress until it had caused a mortality of more than 50% in each of the other two groups, it is clear that thiamine hydrochloride protects foxes from the disease. Before discussing the possible mechanism by which fresh fish could bring about a deficiency of vitamin B_1, we shall present a description of some of the ranch outbreaks observed. It should be noted in reading these brief histories that the disease followed, in all cases, the feeding of fish.

Ranch outbreaks of Chastek paralysis

Our first observations of Chastek paralysis were made in the spring of 1932 on a ranch owned by Mr. J. S. Chastek near Glencoe, Minnesota. Fish was first used in the ration on this ranch on January 21, 1932, when frozen carp was added to the feed in an unknown proportion. The foxes were well until February 16 when they were reported "off feed". The first death occurred 1 week later, on February 23. By March 17 a total of sixty-eight foxes, or 34% of the herd of 208, had died and many of the remaining animals were ill. Because the role of fish as a cause of the disease was unknown at that time, the diet was essentially unchanged until March 17 when fish was removed from the ration and increased amounts of liver were added. After that date only two foxes died. Foxes too ill to eat after March 17 were treated by intraperitoneal injections of saline and by tube-feeding water and milk.

During the winter of 1936 two fox farms near Logan, Utah, experienced outbreaks of Chastek paralysis. On both, frozen carp was used as part of the ration beginning on January 10. On one, the L. ranch, 30% of the diet was carp; and on the other, the R. ranch, 10% was carp. About 1 month later, symptoms of Chastek paralysis developed on both ranches and one death occurred on the R. ranch before February 13, the date

fish was eliminated from the rations of both ranches. As a result of a prompt dietary change, the mortality on these two ranches was relatively low. Of the 544 foxes on the L. ranch, 1.8% died; and of the sixty-nine foxes on the R. ranch, 5.8% died. The diets that induced Chastek paralysis on these ranches are listed in table 1.

In March and April, 1939, an outbreak of Chastek paralysis occurred on a silver fox farm near Grand Forks, North Dakota. A total of 236 foxes (140 females and 96 males) was present on the ranch before the outbreak. Twenty-eight (twenty-two males and six females) died. A complete dietary schedule from

TABLE 1

Diets which caused Chastek paralysis on two fox ranches in Utah, 1936

	L. FOX FARM	R. FOX FARM
	%	%
Fish	30	10
Red meat (horse and beef)	30	40
Tripe		10
Prepared cereal	15	25
Laurel wheat	15	
Carrots	10	15

3 months prior to the outbreak until the disease had entirely disappeared, is shown in table 2.

Feeding Lake Superior herring on 4 days a week and no fish on 3 days a week for nearly 2 months did not in any way harm the foxes or predispose them to Chastek paralysis. The disease first appeared about $3\frac{1}{2}$ weeks after "mullets or suckers from Lake Manitoba" were substituted for the herring and the ration was changed to include fish 7 days a week instead of 4 days a week.

Although the foxes ate poorly after March 12, no deaths occurred until March 27 when one male died. The next day a second male died. A more varied diet, which did not include fish, was adopted on March 28 and was maintained until the disease had completely disappeared. After this change in diet, there was a rapid improvement so that the foxes were soon

eating well. However, the disease had apparently advanced so far in some animals that the change in diet did not cure them. As a result, there were twenty-six deaths from March 30 through April 10. The disease in this instance may be attributed solely to the mullets or suckers fed as 15% of the diet 7 days a week beginning February 15.

TABLE 2

Rations fed on a silver fox farm near Grand Forks, North Dakota, 1938–39

	DEC. 20 TO FEB. 14[1]	FEB. 15 TO MAR. 17	MAR. 18 TO MAR. 24	MAR. 25 TO MAR. 27	MAR. 28 UNTIL AFTER DISEASE DISAPPEARED
Meat	70%	70%	70%	100%	70% [2]
Beef melts		15 lb.			
Horse meat	90 lb.	80 lb.	90 lb.	100%	
Fish [3]	30 lb.	40 lb.	40 lb.		
Horse entrails	15 lb.	20 lb.	20 lb.		
Horse liver		10 lb.	10 lb.		
Beef tripe	10 lb.	15 lb.	15 lb.		
Purina meal	20%	20%	20%		20%
Ground green bone	5%	5%	5%		5%
Lettuce and carrots	5%	5%	5%		5%
Supplementary ingredients					
Milk	3 gal.	5 gal.	2 gal.		3½ gal.
Dry brewer's yeast	3 lb.	3 lb.	3 lb.		3 lb.
Cod liver oil	1 pt.	1 qt.	½ pt.		1 pt.
Dried figs		4 lb.	3 lb.		2 lb.
Fresh eggs		5 doz.	5 doz.		5 doz.

[1] Mixed feed was fed 4 days a week and pieces of horse meat, 3 days a week.
[2] The 70% meat includes all kinds named except fish and horse entrails.
[3] Lake Superior herring was fed until February 14; mullets or suckers from Lake Manitoba were fed from February 15 through March 24.

Chastek paralysis developed during January, 1940, on a ranch near Coon Rapids, Minnesota, where the herd consisted of seventy foxes, or thirty-five pairs. The diet on this ranch was 20 or 30% commercial feed and 70 or 80% rabbit carcasses. On January 15, 1940, carp and northern pike were introduced into the diet as about 50% of the total ration. The remainder of the diet was now 30% rabbit and 20% commercial feed. About 1 week after fish was added, some of the foxes

began to leave part of their feed. The number increased during the following week until practically all the foxes were refusing food. Neurologic symptoms were noted January 24 or 25, and the first death occurred January 26. Fish was removed from the diet at that time and the previous diet of rabbit and commercial feed was resumed. Deaths continued to occur until January 30 when the owner informed us of the epidemic. We immediately went to the ranch to observe the outbreak and to institute treatment.

Three foxes found dead in the pens at the time of our arrival brought the total number of deaths to ten. One other animal, lying on its side unable to rise and apparently moribund was given an intraperitoneal injection of 50 mg. of thiamine hydrochloride and 50 cc. of a 10% glucose solution. Without further treatment, this fox recovered so rapidly that in 12 hours it was able to stand on its feet, although unable to walk without staggering. Subsequently, tube-feeding of eggs, liver juice, milk, water and small amounts of yeast were given, and within 1 week the fox seemed to be entirely normal. It remained in good health and was bred successfully a few weeks later.

At the time of our visit on January 30, five foxes, in addition to the one already referred to, were off feed but showed no definite neurologic symptoms. Each was given an injection of 20 mg. of thiamine hydrochloride subcutaneously and, although one of the foxes had not eaten for 5 days, neither it nor any of the other four developed further symptoms of Chastek paralysis.

On January 31 large amounts of liver (about 30% of the total diet), some yeast and eggs were added to the diet of rabbit meat and commercial feed, and the outbreak soon ceased. While the number of animals available for this therapeutic test of synthetic thiamine hydrochloride was insufficient to permit the simultaneous use of controls, the results of the injections were so favorable that we believe they provide significant confirmatory evidence that the deficiency is really one of vitamin B_1, as seems proved by other evidence.

An opportunity to study in detail the effect of fish in fox rations was provided by extensive feeding trials which were conducted on the three large Fromm Brothers, Nieman and Company ranches near Thiensville, Wisconsin. Following a limited trial of fresh fish in the ration of the foxes during the winter of 1934–35, the feeding of fish in various forms was extended to more than 6700 foxes. Beginning on November 20, 1935, fish in three forms—fresh fish, canned fish and fish meal —was added to the fox ration. About January 1, losses of two or three foxes a day began to occur. Deaths were scattered throughout all the ranches. A few deaths occurred almost daily until January 25, when the number increased to twenty-nine for the 3-day period from January 25 to 27. During the next 3 days, January 28 to 30, fatalities climbed to a total of eighty-three, averaging almost twenty-eight a day. Fish in all forms was eliminated from the diet on January 25. After reaching a high point late in January, the mortalities subsided rapidly to about five a day for a period of 2 weeks. Further losses of two or three foxes a day continued for another 2 weeks with the outbreak ending during the first days of March. In table 3 are listed the ingredients of the diets containing the three types of fish preparations, together with the number of foxes fed these diets and the losses resulting in each group. The fresh fish was Atlantic whiting and the canned fish, Pacific coast mackerel. The symptoms and pathologic findings in foxes dying during the outbreak were diagnostic for Chastek paralysis.

From the data presented it appears that canned fish is less active than is fresh fish in producing Chastek paralysis. However, this is not a certainty, since the species of fish fed in the fresh state was different from that which was canned.

Of about 1200 foxes fed a diet containing 3% fish meal, only 0.6% died. The fish meal used on these ranches is a product so concentrated that 3% used in the rations is considered as equal in nutritive value to about 20% of fresh fish. It is probable that the process of preparing fish meal reduces the ability of that substance to produce Chastek paralysis.

*Effect of Chastek paralysis on developing embryos
and nursing pups*

Two fox farms in Wisconsin, the Swinghammer Fur Farm
and the Howland-Daly Fox Ranch, experienced during the
month of May, 1938, a destruction of a large proportion of
their crop of fox pups from Chastek paralysis. Fish was added
to the ration on the Swinghammer Fur Farm on April 5, and

TABLE 3

*Losses associated with the different diets fed to foxes on three ranches near
Thiensville, Wisconsin, in January, 1936*

	FRESH FISH	CANNED FISH	FISH MEAL
Number of foxes fed	4260	1292	1194
Number of deaths	286	14	7
Loss, in percentage	6.7	1.1	0.6
Complete dietary schedule [1]			
Fish	18.73	13.44	3.11
Liver	1.77	1.72	2.17
Wheat cereal	8.11	9.07	9.35
Oatmeal	4.59	4.90	5.30
Carrots	7.07	7.36	7.17
Lettuce	2.83	2.94	2.49
Horse meat	52.44	56.90	65.80
Eggs	2.20	2.16	2.74
Cod liver oil	0.21	0.22	0.25
Wheat germ oil	0.14	0.11	0.12
Mineral	0.14	0.20	0.25
Dry milk	1.77	0.98	1.25
Total	100.00	100.00	100.00

[1] Amounts of foods in the diets are listed as percentages of the total ration.

on the Howland-Daly Ranch, on April 7. A detailed itemiza-
tion of the diets used on these ranches is presented in table 4.

The population on the Swinghammer ranch before the out-
break of fish disease was eight-five adult foxes and 175 pups.
On May 4 the disease first appeared when fox pups in ten
litters were found to be afflicted. Many more developed the
disease on May 5 and 6. On the latter date a diagnosis of
Chastek paralysis was made and appropriate treatment insti-
tuted. Fish was removed from the diet and increased amounts

of fresh meat, liver, milk and supplements of vitamin B_1 were added to the ration. As a result, the occurrence of new cases was promptly stopped. However, within the short span of 4 days, sixty-two puppies from twenty-five litters had died, a mortality of 35% of all puppies known to be on the ranch at the time.

On the Howland-Daly Ranch only one pup was ill on May 5, but forty-eight, or 33% of all pups on the ranch, had died by May 7, when treatment similar to that employed on the Swing-hammer ranch was instituted and stopped the outbreak.

TABLE 4

Diets which caused Chastek paralysis on the Swinghammer and Howland-Daly Fur Farms

	SWINGHAMMER FUR FARM	HOWLAND-DALY FOX RANCH
	%	%
Horse meat	34.6	38.1
Fish	16.5	5.4
Fish meal		4.3
Cod liver oil	0.7	2.2
Ground green bone		10.9
Gland meat		10.9
Prepared cereal	36.2	21.7
Carrots	9.0	6.5
Raisins	3.0	
Total	100.00	100.00

The effect of Chastek paralysis on pregnancy has been clearly shown in several outbreaks. Markedly decomposed fetuses are usually found in the uteri of pregnant females that have died from Chastek paralysis. It appears certain that the fetus dies several days before the adult fox succumbs. The outbreak of this disease on the fur farm near Grand Forks, North Dakota, occurred at a time when most of the female foxes were pregnant. Although 134 adult females remained alive after the outbreak and showed no residual symptoms, only fifteen of them raised their young. Similar experience on other ranches has shown that a nonfatal attack of Chastek paralysis will regularly cause the intrauterine death of developing fetuses.

DISCUSSION

Since the only factor of apparent epidemiologic significance which is common to all ranches where Chastek paralysis has occurred appears to be the feeding of fresh fish as 10% or more of the ration, and since experimental data substantiate the fact that an otherwise satisfactory ration will produce Chastek paralysis regularly in foxes if fish is added, we feel there can be little doubt that the cause of Chastek paralysis is the feeding of fresh fish. Probably more carp than any other species of fish is used on ranches in the Midwest, and most of the outbreaks of the disease observed by us have been on ranches where carp was fed. In one case it was clear that Chastek paralysis was caused by the feeding of Atlantic coast whiting. On another ranch the fish was reported as being "mullets or suckers from Lake Manitoba". A ranch in Iowa suffered from Chastek paralysis as a result of the feeding of quillbacks from the Cedar River. It is apparent that many diverse kinds of fish, including species from both salt and fresh water, may cause the disease. However, we have some evidence to indicate that certain species, such as fresh water herring from Lake Superior or Michigan, are not nearly so destructive as are carp and the others just mentioned. Experiments now in progress are designed to demonstrate which parts of the injurious fish produce Chastek paralysis and, further, to determine why some fish are less active than others in the production of the disease.

There is clinical and experimental evidence (Perla, '38) obtained from work with human beings and with rats, that if a lactating female subsists on a ration moderately deficient in vitamin B_1, symptoms of a B_1 avitaminosis may develop in the nursing young while the mother remains in apparent good health. The severity of outbreaks of Chastek paralysis observed among nursing pups on ranches where the morbidity and mortality among adult animals was minimal is evidently an analogous phenomenon. There are pathologic, as well as clinical, differences between adult and young foxes with Chastek paralysis which may represent the differences between

the acute and the subacute or chronic disease. To what extent the factor of age, irrespective of acuteness or chronicity, influences the clinical and pathologic manifestation of the disease, we cannot say at this time.

Mechanism by which feeding of fish causes a vitamin-B_1 deficiency

Reported estimates of the amounts of vitamin B_1 found in various kinds of fish and red meats vary from 5 to several hundred international units per 100 gm., depending upon the kind of fish or meat and upon many experimental factors. It is apparent, however, that the expected decrease in the amount of this vitamin resulting from substituting fish for horse meat as 10%, or even as 25%, of the rations fed to foxes would not be enough to cause a B_1 avitaminosis. A small amount of fish may cause a vitamin-B_1 deficiency even though the diet, exclusive of fish, contains a moderate excess of that vitamin.

How fish is able to produce this result we do not yet know with certainty, but there are at least three possibilities. First, fish may somehow increase the animals' need for vitamin B_1 by a metabolic effect; second, fish may prevent absorption of the vitamin; and third, fish may bring about an inactivation or destruction of the vitamin in the food.

The present evidence is compatible with any of these explanations and the third one seems to us to be especially worthy of careful consideration. It appears reasonable to hypothesize that fish may inactivate or destroy the vitamin, if one postulates that small amounts of thiamine (1 mg. or less) found in a normal fox's daily ration may be destroyed, but that if relatively large doses are given, enough remains undestroyed to meet the needs of the fox. It is known that neutral sulfite readily splits thiamine. R. R. Williams ('39), after mentioning this fact, goes on to state: "Barium nitrite or sodium acetate appear, under certain conditions, to effect splitting in a similar sense and *it seems probable that, in neutral foods, free thiamin is very subject to corresponding*

splitting under influences which are as yet imperfectly under-stood.''[5] It may be that some constituent of fish acts as sodium sulfite, barium nitrite or sodium acetate do to inactivate thiamine.

In no case have we observed an outbreak of Chastek paralysis on a ranch where only cooked fish was fed. Apparently, that substance in fish which causes a B_1-avitaminosis in foxes is readily destroyed by heat.

Many of the early descriptions of beriberi were of cases among people in the Dutch East Indies and Japan whose diets consisted largely of polished rice and fish. Possibly the fish, as well as the rice, eaten by these persons may have been a factor in causing the deficiency disease which they developed. It is generally true in regions where fish is abundant that a variety of preparations using pickled or raw fish is commonly eaten.

Cleavage of thiamine by sodium sulfite, sodium acetate, or barium nitrite does not occur in a medium with a high acidity (Williams, '39) and probably the pH of the medium would exert a similar effect if the vitamin were inactivated by foods such as fish. This fact suggests that achlorhydria may influence the production of B_1 avitaminoses in man and lower animals. Obviously, most human patients with achlorhydria do not develop symptoms of B_1 avitaminosis, but in those on a limited diet containing an amount of vitamin B_1 barely sufficient for their needs, the lack of normal gastric acidity may permit destruction of enough of that vitamin to be of clinical importance. Clinical evidence suggests that achlorhydria is a factor in the production of Wernicke's disease.

SUMMARY

Chastek paralysis is an economically important acute disease of foxes occurring in violent outbreaks on ranches where uncooked fish is included as 10% or more of the ration. It causes a high mortality among nursing pups and intrauterine

[5] Italics by present authors.

death of fetuses. The disease may easily be produced experimentally with a diet containing fresh carp, and can be prevented by adding large amounts of thiamine to the same ration. Characteristic neurologic symptoms occur and diagnostic lesions, identical with those of Wernicke's hemorrhagic polioencephalitis of man, are found in the brain. Pathologic findings, limited therapeutic field trials of thiamine and histories of ranch outbreaks confirm the experimental evidence that Chastek paralysis is fundamentally a B_1 avitaminosis.

How fish can induce a deficiency of vitamin B_1 is unknown, but it is suggested there may be a chemical splitting of thiamine by some constituent of fish.

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THE SPECIFIC DYNAMIC EFFECTS OF AMINO ACIDS AND THEIR BEARING ON THE CAUSES OF SPECIFIC DYNAMIC EFFECTS OF PROTEINS [1]

MAX KRISS

Institute of Animal Nutrition, The Pennsylvania State College, Pennsylvania

Since Lusk ('10, '12) first published his experiments on the influence of ingestion of amino acids on metabolism this subject received the attention of many investigators, and the experimental results obtained were often variously interpreted as explanations of the causes of specific dynamic effects of proteins. Some idea as to the complexity of the problem and of the scope and volume of the work accomplished may be obtained from the reviews of the literature by Lusk ('31 a), Grafe ('16), Mitchell and Hamilton ('29), Lundsgaard ('31), Borsook ('36), Brody and Procter ('33) and Wilhelmj ('35).

In spite of these extensive researches considerable confusion exists as to the specific dynamic values of the different amino acids and as to their bearing on the causes of specific dynamic effects of protein. This confusion of ideas, which is due mainly to the diversity of experimental results, is epitomized in the following words of Lusk ('28): "We may now proceed in greater detail into the clouded area of the cause of the specific dynamic action of protein. . . . The hypotheses which have been presented cannot now be welded into a concordant whole. They transcend one's powers to coordinate them."

The following few specific references may serve to illustrate the diverse character of the experimental results and of their interpretations.

Thus, when Lusk ('12) found that glutamic acid administered to a dog was without effect on the heat production while the amino acid itself was metabolized, he concluded that the process of deaminization and urea formation must be ruled out from being a cause of the specific dynamic effect of protein. These observations and conclusions were later confirmed by Chambers and Lusk ('30). Grafe ('16), however, found that after the administration of glutamic acid to a dog the oxygen consumption was appreciably increased, and on the basis of his experiments with this and other amino acids he arrived at the conclusion that the specific dynamic effect of protein was largely due to the liberation of the NH_2 radicle of amino acids.

Glycine and alanine were found by Lusk ('12, '15) to exert pronounced effects on metabolism, the increase in heat production caused by glycine being considerably greater than that caused by alanine. These results were originally viewed by Lusk as support for his theory of cell stimulation. Aubel ('28) explained the specific dynamic effect of alanine by the transformation of this substance to glucose, and very recently Oberdisse ('39) concluded from his experiments with alanine administered to dogs intravenously that the specific dynamic effects of amino acids were due almost entirely to the nitrogen-free deamination products which are oxidized after conversion into carbohydrate.

Aspartic acid and asparagine showed no specific dynamic effect in the experiments of Atkinson and Lusk ('18); and the same significance was attached by the authors to this finding as to the negative results obtained with glutamic acid.

Lundsgaard ('31) observed that glycine, alanine, glutamic acid, aspartic acid and tyrosine all produced dynamic effects, and that these effects were in definite relationship with the amounts of nitrogen of administered amino acids.

Lusk's ('31 b) final view on this subject was in conformity with Rubner's ('02) general hypothesis that the specific dynamic effect of protein is due to the free heat of intermediary thermochemical reactions.

Viewing the available evidence as a whole one can hardly escape the conclusion that the question as to whether or not amino acids have definite specific dynamic values still remains unsettled. The numerous diverse data already on record seem to hold little promise that any further investigation of this subject, by the procedures thus far followed, will yield results of general uniformity and significance.

In the interest of further advance in this field of research new angles of approach to the problem of evaluating the dynamic effects of amino acids must be sought with a view of eliminating possible disturbing factors. This involves the question of procedure. The experiments presented herein represent an effort in this direction.

More specifically, this paper reports determinations of the specific dynamic effects of the amino acids glycine, alanine, glutamic acid, aspartic acid, asparagine and tyrosine by a procedure which constitutes a radical departure from the methods generally followed in the past work with amino acids.

DISCUSSION OF METHODS

The method employed in this investigation for the determination of the specific dynamic effects of the amino acids is an adaptation of the method developed by Kellner ('00) and by Armsby (Armsby and Fries, '03) for measuring the energy metabolism in relation to the diet. Most, if not all, of the past determinations of the specific dynamic values of amino acids reported in the literature were based on Rubner's method of measuring the specific dynamic effects of nutrients. There are at least two important differences between these two classical methods which need emphasis:

First, according to Rubner's procedure the test substance is administered only once and the heat measurement is begun soon after the substance is administered. If the period of

observation is short, as it was in most of the published experiments with amino acids, there is always a question as to what fraction of the ingested material is represented by the observed increase in heat production. According to the method of Kellner and of Armsby the heat measurement is not begun until after the animal has been on the experimental diet for a number of days, and thus it is likely to be well representative of the diet.

The other important difference between the methods is in the base values used for the determination of heat increments (dynamic effects) of feeding. The fundamental characteristic feature of Rubner's method is the use of the heat production of fast as the base value. The method used by Kellner features maintenance metabolism as basal. Armsby determined heat increments of feeding between various planes of nutrition, including submaintenance planes. It is, however, a significant fact that in a long series of calorimetric experiments, which were last planned by Armsby (see Fries, Braman and Cochrane, '24; Cochrane, Fries and Braman, '25), the heat production of maintenance was prominent as a base.

A question of crucial importance in the use of Rubner's method is whether the body nutrients katabolized during fast do not themselves exert dynamic effects. This question received relatively little attention in the past, a negative answer, that is, the non-existence of dynamic effects of body nutrients, being quite generally tacitly assumed. The significance of an affirmative answer to the foregoing question would be to the effect that the increase in heat production over the fasting metabolism, resulting from food consumption, is not to be taken as a measure of the true or absolute dynamic value of the food nutrients ingested, but as representing the difference between the dynamic effects of the ingested food nutrients and those of the body nutrients spared.

From the nature of the problem no direct proof of the existence or non-existence of dynamic effects of katabolized body nutrients seems possible. There is, however, sufficient indirect affirmative evidence in the literature to justify the considera-

tion of dynamic effects of body nutrients as a strong possibility, if not an established fact. Very briefly the evidence is along the following lines:

1. Increase in heat production accompanying an increase in protein break-down during fast (Rubner, '02; Chambers and Lusk, '30; Dann, Chambers and Lusk, '31).

2. The very low and much more variable heat increment values of rations and single nutrients below maintenance, with fasting heat production as a base, and with concomitant sparing of body nutrients, as compared with the higher and less variable heat increments caused by the addition of the same food substances to a maintenance ration (Richardson and Mason, '23; Forbes, Braman and Kriss, '28, '30; Kriss, Forbes and Miller, '34; Kriss, '38).

3. Thermochemical considerations. If the intermediary reactions and energy exchanges in the metabolism of food nutrients are accompanied by increased heat production (Adams, '26; Aubel, '28; Borsook and Winegarden, '31; Lusk, '31 b, and others), it is hardly conceivable that all the transformations incident to the katabolism of body nutrients during fast should be accomplished without energy expenditure. According to Borsook ('36) "there is no reason to expect different energy changes whether tissue protein and amino acids or amino acids immediately derived from ingested protein are metabolized."

4. Similarity between heat increment values of protein (casein), carbohydrate (starch) and fat (olive oil) determined with respect to fasting heat production as a base but corrected, by a conventional procedure, for the sparing of body nutrients, and the heat increment values of the same food nutrients determined by the use of the heat production of maintenance as a base (Kriss, Forbes and Miller, '34).

In view of all the foregoing considerations we feel justified in our departure from the use of the fasting heat production as a base value in our present study of the specific dynamic effects of amino acids, and in our attempt to follow the method embodying the principle of Kellner and Armsby, as elaborated

by Kriss, Forbes and Miller ('34) and as applied more recently to the determination of specific dynamic effects of different proteins (Kriss, '38).

The procedure followed in this investigation was, in most respects, the same as that used previously in the study of the specific dynamic effects of proteins (Kriss, '38). The experimental subjects were thirty-two male rats weighing approximately 200 gm. each. The same rats served as the subjects in previously reported studies of nitrogen, carbon and energy balances (Kriss, '39; Kriss and Marcy, '40 a), which were carried out in connection with and as part of the present study.

All rats received as their first experimental dietary treatment 8 gm. per day of a basal ration (as in the studies last cited) which was adequate for the maintenance of approximate equilibrium of nitrogen and energy. As in the previous experiments, the daily allowance of food was given in two equal portions, one in the morning and one in the evening. At the end of approximately 3 weeks on this diet the rats were subjected to respiration trials for the measurement of the total energy metabolism.

Following this treatment the rats, divided into groups, received, in addition to the basal ration, supplementary portions of different amino acids as the test substances. It was intended to feed all the supplements in quantities supplying 7500 calories of gross energy per day. Some of the amino acids, however, could not be consumed by the rats in such large quantities, and, consequently, were given in smaller amounts. The number of rats in each group, and the kind and quantity of supplement fed to each rat were as follows: Five rats received 2.06 gm. per day of d-glutamic acid. Six received 1.74 gm. of dl-alanine. Five received 1.8 gm. of glycine; one of this group (rat 11) received, in a different period, 2.4 gm. of glycine. Six received 1.288 gm. of l-tyrosine.

Five received 2 gm. of l-aspartic acid. Five received 2 gm. of l-asparagine.

The amino-acid-supplemented diets were fed to the rats for a period of 8 days. At the end of this period the rats were again subjected to the measurement of the total respiratory exchange. This was followed by a 7-day feeding period on the basal ration and another respiration experiment at the end of this period. Finally, after continued feeding on the basal ration for 7 days the rats were subjected to a 24-hour fast and to the determination of the fasting heat production in a 6- to 7-hour period. These determinations were used as a basis for correcting the total heat production of the animals for differences in body weight.

The respiratory measurements in the feeding periods began soon after the morning meal was consumed and continued for 7 consecutive hours. These measurements were used as a basis for the comparisons between the heat production of maintenance and supermaintenance feeding.

The apparatus used for measuring the respiratory exchange, and the general technic employed were as described in the previously cited publication (Kriss, '38).

The metabolism of protein and of amino acids was accounted for in the computation of the total heat production in the feeding periods on the basis of the urinary nitrogen excretions and the respiration and energy factors reported by Kriss ('39) and Kriss and Marcy ('40 a).

The practice of computing the heat production, for comparative purposes, to a basis of uniform empty body weight was continued. To this end the alimentary fill was determined, with each dietary treatment, in control animals which were comparable in weight with the experimental subjects.

DISCUSSION OF RESULTS

In the discussion of the results of the present experiments comparisons are made of certain of our data with results obtained by other investigators. These comparisons often bring out certain conflicting ideas. In considering these con-

trasts the reader should not lose sight of the differences in experimental procedure which were pointed out in the early part of the paper.

The data representing the average hourly heat production of the individual rats, per 200 gm. of empty body weight, and the calculated increases in heat production resulting from the addition of the various amino acid supplements to the basal ration are represented in table 1.

The uniformity of the heat production representing the basal ration is noteworthy. The average results of each group show only slight differences in metabolism between the initial and final maintenance periods. In accord with previous practice, the average heat production for the two maintenance periods (one immediately preceding and one immediately following the supermaintenance period) was used as the base value for the computation of the heat increments due to the supplements. It is believed that this procedure satisfactorily compensates for any slight changes in the metabolism incidental to the changes in age and body weight.

The heat production representing the amino acid-supplemented rations also shows a fair degree of uniformity within each group, and, without a single exception, is found to be higher than the corresponding value for the basal ration.

The increases in heat production of the individual rats, resulting from the administration of the amino acids, are presented in the last two columns of table 1 and are expressed as calories per hour and as per cent of metabolizable energy. The last-mentioned data served as the basis for the determination of the significance of differences between the specific dynamic effects of the different amino acids, which will be discussed later. The metabolizability of the amino acids was determined for each rat on the basis of the work reported by Kriss ('39) and by Kriss and Marcy ('40 a).

The variability of the results for heat production and for heat increments, within each group, is indicated by the respective values for standard deviation, which are self-explanatory. For the purpose of comparing the dynamic effects

TABLE 1

Hourly heat production of rats per 200 gm. of empty body weight as influenced by the addition of amino acids to a basal maintenance ration

RAT NO.	BASAL RATION, 8.0 GM. DAILY			BASAL RATION PLUS AMINO-ACID SUPPLEMENT	INCREASE IN HEAT PRODUCTION DUE TO AMINO ACID	
	Initial period	Final period	Average of the two periods	2.06 gm. glutamic acid	Total	Per cent of metabolizable energy
	cal.	*cal.*	*cal.*	*cal.*	*cal.*	
1	820	868	844	1020	176	71.3
2	854	875	865	1020	155	60.2
3	848	816	832	1015	183	81.3
4	801	825	813	990	177	67.1
5	787	781	784	955	171	62.5
Average	822	833	828	1000	172	67.9
Standard deviation	±28.7	±38.9	±30.8	±28.1	±10.6	±8.3
				2.4 gm. glycine		
11	813	886	850	973	123	61.5
				1.8 gm. glycine		
11	813	886	850	938	88	55.4
12	788	835	812	926	114	71.3
13	906	860	883	965	82	53.3
14	829	825	827	890	63	40.9
15	849	810	830	913	83	52.2
Average	837	843	840	926	86	54.8
Standard deviation	±44.6	±27.1	±27.4	±28.0	±18.2	±10.9
				1.74 gm. alanine		
21	829	805	817	939	122	59.9
22	841	785	813	947	134	66.4
23	821	791	806	969	163	77.9
24	830	797	814	932	118	55.3
25	878	838	858	980	122	61.4
26	894	810	852	946	92	42.4
Average	849	804	827	952	125	60.3
Standard deviation	±32.5	±18.1	±22.3	±18.5	±23.2	±13.6
				1 288 gm. tyrosine		
41	857	815	836	923	87	51.1
42	888	833	861	976	115	58.4
43	890	829	860	938	78	44.9
44	877	804	841	917	76	41.1
45	861	828	845	950	105	55.1
46	932	886	909	995	86	44.6
Average	884	833	859	950	91	49.2
Standard deviation	±27.1	±28.3	±26.7	±30.5	±15.5	±6.8
				2.0 gm. aspartic acid		
51	882	887	885	950	65	32.9
52	905	872	889	958	69	34.1
53	938	890	914	981	67	37.2
54	908	873	891	985	94	59.2
55	863	823	843	940	97	53.4
Average	899	869	884	963	79	42.4
Standard deviation	±28.4	±27.0	±25.8	±19.6	±15.7	±12.1
				2.0 gm. asparagine		
56	892	845	869	962	93	53.7
57	884	863	874	986	112	67.5
58	882	854	868	941	73	51.1
59	953	921	937	999	62	37.1
60	851	850	851	939	88	51.2
Average	892	867	880	965	85	51.8
Standard deviation	±37.3	±31.1	±33.1	±26.7	±19.2	±10.8

of the different amino acids, the average heat increment of each group was used.

The administration of glutamic acid resulted in pronounced and relatively uniform increases in heat production. These results are in contrast to the negative findings of Lusk ('12) and Chambers and Lusk ('30), and substantiate the positive dynamic effects of this amino acid observed by other investigators (Grafe, '16; Lundsgaard, '31; Luck and Lewis, '34).

Borsook ('36) suggested that the probable reason for Lusk's failure to observe any specific dynamic effect with glutamic acid was the short period of observation and the slow absorption of this amino acid from the intestine. In the present experiments glutamic acid constituted a part of the animals' diet for a period of 8 days and it was fairly completely absorbed (about 95%). In the light of the previous discussion of the question of technique, these experiments leave little doubt that the positive heat increments possess the greater significance.

Of similar significance are the positive increases in heat production caused by the administration of aspartic acid and asparagine. It will be recalled that these substances exhibited no significant dynamic effects in the experiments of Atkinson and Lusk ('18).

Glycine and alanine, administered in approximately equi-caloric quantities, caused approximately the same hourly increases in heat production. The heat increments of equi-caloric quantities of tyrosine were smaller. Tyrosine was less completely metabolized than were the other amino acids.

Different quantities of glycine supplement (2.4 gm. and 1.8 gm. per day) fed in different periods to the same animal (rat 11), produced nearly proportionate increases in heat production (51 calories and 49 calories per gram, respectively). This is interesting, since, in similar experiments with proteins (casein, gelatin and beef muscle) reported by Kriss ('38) nearly proportionate increases in heat production were observed when these substances were fed in quantities of 1.5 gm. and 3.0 gm. as supplements to a basal ration.

There is also some evidence (Forbes, Braman and Kriss, '28, '30) to the effect that the heat increments of mixed rations, above the maintenance level, are approximately proportional to the increases in food consumption within fairly wide limits.

TABLE 2

Specific dynamic effects of amino acids expressed in various ways

CATEGORY OF INTEREST	DAILY SUPPLEMENT							
	Glutamic acid 2.06 gm.	Alanine 1.74 gm.	Glycine 2.4 gm.	Glycine 1.8 gm.	Tyrosine 1.288 gm.	Aspartic acid 2.0 gm.	Asparagine 2.0 gm.	Coefficient of variation in per cent
Number of animals	5	6	1	5	6	5	5	
Metabolizable energy (Cal.)	6.08	4.98	4.80	3.77	4.44	4.47	3.94	
Specific dynamic effects (Cal.):								
Per 24 hours	4.13	3.00	2.95	2.06	2.18	1.90	2.04	
Per gram amino acid fed	2.00	1.72	1.23	1.15	1.70	0.95	1.02	29.2
Per gram nitrogen fed	21.50	11.11	6.66	6.22	22.29	9.16	5.57	60.8
Per gram extra urinary nitrogen	28.87	13.27	7.10	6.88	19.16	13.08	6.71	60.1
Per gram amino acid metabolized	2.83	2.91	1.44	1.37	2.73	1.43	1.46	37.2
Per millimol amino acid metabolized	0.42	0.26	0.11	0.10	0.49	0.19	0.19	59.8
Per gram of carbon of amino acid metabolized	6.93	7.19	4.49	4.26	4.57	3.95	4.01	27.5
Per 100 Calories of amino acid metabolized	92.3	82.0	69.1	65.1	49.2	63.4	67.1	19.9
Per 100 Calories of gross energy	54.9	39.6	39.7	37.0	29.1	32.8	33.9	21.8
Per 100 Calories of metabolizable energy	67.9	60.3	61.5	54.8	49.2	42.4	51.8	15.4

Since much of the explanation of the causes of specific dynamic action of proteins logically depends on the specific dynamic effects exhibited by different amino acids, the manner of expressing these effects obviously plays an important role.

With this in mind an attempt was made to bring out certain important relationships by expressing the results of these experiments in several different ways as presented in table 2.

The specific dynamic values expressed as Calories per gram of amino acid fed or as Calories per gram of amino acid metabolized show considerable variability. This is entirely expected in consideration of the different chemical structure and different metabolic paths of these substances. On either basis the dynamic values for glutamic acid, alanine and tyrosine are considerably higher than the corresponding values for glycine, aspartic acid and asparagine. The dynamic values for the latter two substances are nearly identical in spite of the marked difference in their nitrogen content.

Lack of a definite direct correlation between the specific dynamic effects of the amino acids and nitrogen metabolism is strikingly shown by the results expressed as Calories per gram of nitrogen fed, as Calories per millimol of amino acid metabolized, and especially, by the values expressed as Calories per gram of extra urinary nitrogen. All these three sets of values are characterized by very high coefficients of variation (about 60%). An inverse relationship is found to exist between the specific dynamic values and the nitrogen content of the amino acids in contrast to the direct correlation between these values observed by Lundsgaard ('31) for glycine, alanine, tyrosine, glutamic acid and aspartic acid. Obviously these methods of expressing the specific dynamic effects of amino acids do not contribute to uniformity. The results suggest that nitrogen metabolism and excretion apparently play a relatively insignificant role in causing the dynamic effects of amino acids.

It is probably significant that the dynamic effects are much more closely correlated with the carbon of the amino acid metabolized than with the extra nitrogen excreted in the urine. The dynamic values expressed per gram of urinary nitrogen vary from 6.71 Calories to 28.87 Calories; while those

expressed per gram of carbon metabolized vary only from 3.95 Calories to 7.19 Calories. The coefficient of variation of the former is 60.1% as compared with the coefficient of variation of 27.5% for the latter.

The figures for the dynamic effects per gram of carbon metabolized are smaller than the corresponding values per gram of urinary nitrogen. This difference in magnitude between the two sets of data is accounted for by the fact that the carbon content of the amino acids is several times their nitrogen content. It can in no way be taken to mean that the increase in heat production arising from the metabolism of carbon is less than that caused by the metabolism and excretion of nitrogen.

Borsook ('36) recently put forth the theory that "the specific dynamic effect of protein is a composite of two factors, one nearly constant, representing the increased energy production attending the metabolism and excretion of the nitrogen and amounts to 7 to 10 Calories per gram of nitrogen; the other —more variable, and at times larger fraction—arises from the metabolism of carbon." The results of the present investigation support the view that the latter factor is the much more important one of the two in this relation. The data do not preclude the possibility that metabolism of nitrogen (deaminization and urea formation) contributes some fraction to the increase in energy production; but they indicate that with rats this fraction is relatively very small—probably considerably smaller than Borsook's estimate. The total specific dynamic effect of glycine—the amino acid containing the highest percentage of nitrogen—is approximately 7 Calories per gram of extra urinary nitrogen. This value includes the increase in heat production arising from the metabolism of carbon. Very recently Kriss and Marcy ('40 b) reported evidence to the effect that excretion of nitrogen in the form of urea is not accompanied by appreciable expenditure of energy.

A further noticeable gain in uniformity of the dynamic values of the different amino acids is shown by the results

expressed per 100 Calories of amino acid metabolized (coefficient of variation equals 19.9%). Apparently the relationship between the energy metabolized and the specific dynamic effects of the amino acids is a more significant one than is either relationship between grams of nitrogen or grams of carbon metabolized and increased heat production. Metabolized energy comprises all the chemical energy of the test substance which was transformed in the body into heat. This was computed by the use of the energy factors determined by Kriss ('39) and Kriss and Marcy ('40 a). Apparently the heat increment of the non-nitrogenous (deaminized) fraction of the amino acid molecule is higher than the heat increment of the nitrogenous fraction.

A comparison of the dynamic values expressed as per cent of the gross energy with those expressed as Calories per gram of nitrogen fed indicates that the increase in heat production is more closely related to the potential energy of the amino acids than to their nitrogen content.

The smallest variation is shown by the dynamic effects expressed as per cent of metabolizable energy (gross energy minus energy of feces and urine, as determined directly), given in the last column of table 2 (coefficient of variation equals 15.4%). Of the latter values, that for glutamic acid is highest and that for aspartic acid is lowest. The dynamic values for alanine and glycine fed in equicaloric quantities are practically identical, but for the smaller quantity of glycine the dynamic effect as per cent of metabolizable energy is somewhat lower than for the larger quantity. Tyrosine and asparagine show practically the same dynamic effects as per cent of metabolizable energy, while their dynamic values per gram of nitrogen fed or per millimol of amino acid metabolized show the greatest difference.

The degree of correlation between the specific dynamic effects of the amino acids and the various metabolic factors may be seen best from the following results of statistical treatment of the data:

Coefficient of correlation between S.D.E. and—(1) metabolizable energy = 0.94; (2) gross energy = 0.70; (3) metabolized energy = 0.68; (4) metabolized carbon = 0.49; (5) millimols of amino acid metabolized = —0.24; (6) nitrogen ingested = —0.53; (7) extra urinary nitrogen = —0.60.

In order to ascertain the statistical significance of the differences among the dynamic effects of the different amino acids, expressed as per cent of metabolizable energy, the values obtained for the different amino acids with the individual animals (table 1, last column) were compared and subjected to a statistical analysis. The results of this analysis have revealed that, of the fifteen possible comparisons made, only three showed differences which were statistically significant. These three comparisons are between glutamic acid, on the one hand, and aspartic acid, asparagine and tyrosine, respectively, on the other. The physiological significance even of these three differences is, however, not certain.

It appears, therefore, that the specific dynamic effects of various amino acids, determined by the use of the heat production of maintenance as a base value, are best correlated with the metabolizable energy of the administered amino acids.

The results are consistent with the theory that the dynamic effects of amino acids, and therefore of proteins, are byproducts of intermediary chemical reactions (oxidative and synthetic) and energy changes, and they do not lend support to the idea that certain amino acids, or certain of their cleavage products act in the body as special metabolic stimulants in the pharmacodynamic sense.

SUMMARY AND CONCLUSIONS

The specific dynamic effects of glutamic acid, glycine, alanine, tyrosine, aspartic acid and asparagine were determined by feeding these amino acids to rats in considerable quantities (some equicaloric) as supplements to a basal maintenance ration. The increases in heat production due to the supplements were measured with respect to the heat production of maintenance as a base value.

All amino acids showed positive dynamic effects. The results were expressed in ten different ways, and a study was made of the relationships between the heat increments and the various metabolic factors.

The heat increments expressed in relation to ingested or urinary nitrogen, as well as the values expressed per millimol of amino acid metabolized, were found to be highly variable, and showed no direct correlation between the specific dynamic effects of amino acids and nitrogen metabolism.

The results expressed as Calories per gram of carbon of the amino acids metabolized were much more uniform than those expressed per gram of extra urinary nitrogen, thus indicating that the metabolism of carbon is probably a more important factor in the production of the specific dynamic effects than is the metabolism of nitrogen.

The closest correlation was found between the dynamic effects and the metabolizable energy of the amino acids.

The results are consistent with the theory that dynamic effects of amino acids, and therefore of proteins, are by-products of intermediary chemical reactions (oxidative and synthetic) and energy changes, and they do not lend support to the idea that certain amino acids or certain of their cleavage products act in the body as special metabolic stimulants in the pharmacodynamic sense.

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SOME OBSERVATIONS OF VITAMIN B_6 DEFICIENCY IN THE DOG[1]

HAROLD R. STREET,[2] GEORGE R. COWGILL AND H. M. ZIMMERMAN

*Departments of Physiological Chemistry and Pathology, Yale University
School of Medicine, New Haven, Connecticut*

THREE FIGURES

Relatively little is known about the nutritional significance of vitamin B_6 in species other than the rat, although recent reports indicate that it is an essential for certain other animals such as the chick (Hegsted et al., '39), pigeon (Carter and O'Brien, '39), dog (Fouts et al., '39) and pig (Chick and associates, '38). The present paper is a report of observations made on dogs allowed to subsist on an artificial diet planned at the time to be as far as possible deficient with respect to vitamin B_6.

EXPERIMENTAL PROCEDURE

Diet employed. The percentage composition of the basal mixture was as follows: vitamin-free casein[3] 25; sucrose 48; hydrogenated cottonseed oil[4] 20; bone ash 4; salt mixture[5] 3. In addition each dog received daily, per kilogram of body weight, 10 µg. thiamine chloride,[6] 25 µg. riboflavin,[7] and filtrate

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[3] Labco brand casein, obtained from the Borden Company, Bainbridge, New York.

[4] Crisco.

[5] Slightly modified Osborne-Mendel salt mixture described by Street and Cowgill ('39).

[6] From the Winthrop Chemical Co., New York, N. Y.

[7] Obtained from the S.M.A. Corp., Cleveland, Ohio.

factor concentrate equivalent to 0.4 gm. liver extract. Vitamins A and D were provided in a cod liver oil concentrate available in the form of tablets, two tablets per dog daily.[8] Our experiments were almost finished when the discovery of the potency of nicotinic acid against canine blacktongue was announced. However, we believe that our animals were receiving nicotinic acid because of the way we prepared our concentrate of the filtrate factor from liver powder,[9] and because of the observation that our first administration of the concentrate in amounts equivalent to 0.2 gm. liver extract per kilogram body weight daily resulted in symptoms suggestive of incipient blacktongue, which symptoms disappeared when the dosage of this concentrate was doubled. In addition to the supplements just mentioned each control animal received daily a vitamin B_6 concentrate in the amount of 0.5 cc. per kilogram of body weight.[10]

Methods. Eight adult mongrel dogs, from $4\frac{1}{2}$ to 9 kg. in weight, were used. They may be classified into three groups according to their dietary regimens.

Group I. Four animals (dogs 31, 33, 37 and 43) were given the basal diet lacking vitamin B_6. Each was offered daily an amount of food furnishing 80 calories per kilogram of body weight. As will be described below, three of these animals were given vitamin B_6 concentrate for short periods, beginning on the 122nd to the 356th day of the experiment, in order to study the curative effect of vitamin B_6 on some of the deficiency symptoms that had developed.

[8] Kindly furnished by the White Laboratories, Newark, New Jersey.

[9] One kilogram of liver extract powder, obtained through the courtesy of Dr. H. W. Rhodehamel of the Lilly Research Laboratories, was dissolved in 7.5 liters of water, the solution brought to approximately pH 4.0 with hydrochloric acid, and then 500 gm. of fullers' earth added with stirring. The earth was removed by decantation and filtration, and the process repeated with three more treatments using 500-gm. portions of the earth each time.

[10] This was prepared from tikitiki (Street and Cowgill, '39) by adsorption on fullers' earth and elution with alkali, as described by Lepkovsky and co-workers ('36) and was brought to a final concentration such that 1 cc. represented 3 gm. of rice polish. We have found concentrates prepared in this way to be potent sources of vitamin B_6 by rat assay.

Group II. Two dogs (nos. 32 and 34) in this group served as inanition controls for dogs 31 and 33, respectively, of group I; each control was given daily the amount of the basal mixture eaten by its experimental partner the preceding day. Dog 38 served as an ad libitum control: it was given the full allotment of 80 calories of the basal mixture per kilogram daily. In addition to the basal diet the control animals were given 0.5 cc. per kilogram per day of the vitamin B_6 concentrate.

Group III. This group is represented by a single animal, dog 36, which served as an ad libitum control for 348 days, receiving 0.5 to 1.0 cc. per kilogram of the vitamin B_6 concentrate daily. Because the dog appeared to have a higher requirement for vitamin B_6 than the other controls, vitamin B_6 was withdrawn on the 348th day to see if deficiency symptoms would develop.

Each dog was given a vermifuge before being placed on the experiment. The animals were weighed weekly and examined frequently for any changes in behavior or appearance. In the latter part of the experiment, after abnormalities of gait had appeared in some of the subjects, all were taught to traverse an obstacle board and to tread on a revolving table, so that changes in neuromuscular control could be seen more readily.

Weekly hemoglobin analyses, by the method of Newcomer ('19), and red blood cell counts were made on all animals. Occasional determinations were made of the free and combined acid in the gastric juice after the stimulation of gastric secretion by histamine or by means of a 7% alcohol test meal, as described by Cheney ('28).

The experimental dogs were autopsied immediately after they had succumbed to nutritional deficiency; the control animals were chloroformed and autopsied when their experimental companions died. Sections of the brain, spinal cord, peripheral nerves, liver, spleen and kidney were preserved for histologic examination, using the same techniques and staining methods as in our previously reported study.

RESULTS

General behavior of the animals. Within 4 to 5 weeks the appetites of the four animals not receiving vitamin B_6 declined and became erratic. However, anorexia never became complete as it so often does, for example, in vitamin B_1 deficiency. Concomitant with the decreased food consumption, the body weight declined gradually. The losses for these four dogs at the lowest point of their weight curves were 25, 36, 36 and 44% respectively, of the original weight. The four control animals appeared to have excellent appetites, always eating their food allotments quickly. Although two of these dogs were restricted to the food intakes of their experimental companions, none of them lost as much weight as the latter animals. Their weight losses were 10, 13, 13 and 15% of their initial weights, respectively.

Intermittent diarrhea occurred in the dogs of group 1; infrequently there was blood in the feces. This diarrhea was most pronounced in dog 37, in which it was present during about one-fourth of the days of the experiment. Thus, in contrast to our observations of canine riboflavin deficiency (Street and Cowgill, '39) and of chronic vitamin B_1 deficiency,[11] diarrhea is a significant feature of vitamin B_6 deficiency in dogs. On the other hand, vomiting was rarely seen in this experiment; none of the dogs was observed to vomit more than once or twice throughout the entire experimental period. The control animals did not suffer from diarrhea.

Gait. Abnormalities of gait appeared in three of the animals in group 1; dog 43, which did not survive as long as the others, did not show this change. In the case of dog 31 it was observed on the 231st day that the hind legs were stiff and that the animal stood with the hind legs far apart ("wide base"). The condition soon became more marked, and the gait so uncertain that the animal would fall over when it attempted to shake itself. Dog 33 showed changes in gait of the same type which, however, were much less marked; this was first noticed on the

[11] Unpublished observations.

245th day as a stiffness of the hind legs. By the 312th day there was a swaying of the hind quarters in walking. Abnormal gait appeared in dog 37 on the 217th day as a slightly spastic, slightly swaying gait. This abnormality became very gradually more marked until it was rather striking by the 434th day. At this time there was slight stiffness of the hind legs, wide base, and an awkward "compass gait", the hind legs swinging out wide at each step.

These dogs were examined several times by Dr. James C. Fox, Jr. An illustration of the observations made in this connection is seen in the following extracts of the reports pertaining to dog 37.

Dog 37--314th day. "The behavior on the obstacle board is odd. There is overstepping with the hind legs and prolongation of balancing on the forepaws. The dog is stable on the turntable, or may slip a little more than normal. Balances well on the hind legs. The deep reflexes are all normal."

423rd day. "After watching the dog's behavior the definite impression was gained that he was unsteady and ataxic on standing, but not weak. His hind legs overthrow on the obstacle board. He is uneasy on the turntable, preferring not to tread. Does not tire or slump when standing on his hind legs. The deep reflexes are lively. *Impression:* Posterior column degeneration."

As the syndrome of vitamin B₆ deficiency advanced the dogs became quieter, and sometimes weak and reluctant to walk. The administration of vitamin B₆ in the form of a concentrate (see figs. 1, 2 and 3) always brought about a return to the usual activity within a few days. The appetites of the experimental dogs also increased more or less promptly following treatment with vitamin B₆ concentrate.[12] In one of the instances in which the authors' B₆ concentrate was given, the appetite returned to normal within 24 hours; in two other instances a few days were required for complete restoration of the appetite. In two cases in which Merck's B₆ concentrate was given the appetite became normal within 24 hours, in the third case within 48 hours. The administration of Lepkovsky's

[12] Two different vitamin B₆ concentrates were used, namely, that prepared by the authors and a preparation kindly sent to us by Dr. W. L. Sampson of the Merck Institute of Therapeutic Research. The latter concentrate was reported to be entirely free of thiamine, riboflavin and filtrate factor.

('38) crystalline factor 1 to one animal (fig. 3) did not lead to any clear effect on food consumption or the degree of activity.

In contrast to the quietness and abnormal gait of the experimental dogs, the control animals of group 2 remained lively and vigorous and free from neuromuscular defects.

Hemoglobin and erythrocyte studies. Weekly blood analyses disclosed a gradually developing microcytic, hypochromic anemia, which became severe in three of the experimental dogs. In the fourth experimental animal (dog 43) the hemoglobin had fallen from an initial value of 15 gm. per 100 cc. to
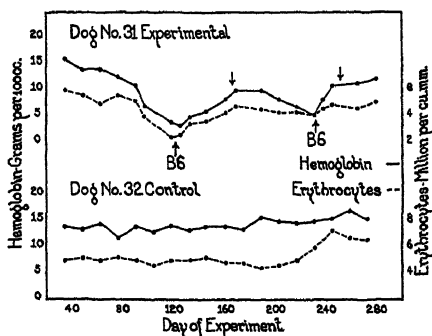
Fig. 1 Hemoglobin and erythrocyte levels of dog 31 and its inanition control mate. At the first arrow ($\nearrow$ B_6) the administration of the authors' vitamin B_6 concentrate was begun at the level of 1.25 cc. daily. This was given for 31 days, followed by 2.50 cc. for 12 days. At the second arrow ($\nearrow$ B_6) the Merck B_6 concentrate was given, fifteen single rat doses per day for 20 days. The inverted arrows ($\searrow$) indicate the withdrawal of the vitamin B_6 supplement.

9 gm. at the time of death. The hemoglobin and erythrocyte levels of the four control animals remained within the normal range with the exception that dog 36 showed a transitory anemia in the early part of the experiment, the hemoglobin falling to 6 gm. per 100 cc. The data for some of the animals are shown graphically in figures 1, 2 and 3. It will be observed that the hemoglobin of the animals of group 1 fell to levels of approximately 5 gm. per 100 cc. within from 120 to 320 days. When the anemia had reached this stage, treatment was instituted with vitamin B_6 preparation or, in one case, with ferrous sulfate. The B_6 concentrates and the ferrous sulfate were
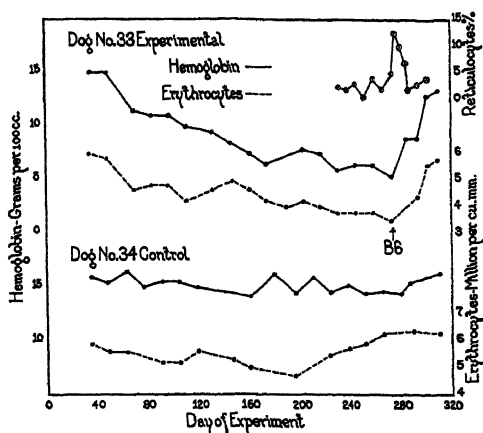
Fig. 2   Hemoglobin and erythrocyte levels of dog 33 and control mate. The arrow indicates the beginning of treatment with the Merck vitamin B_6 concentrate. Fifteen single rat doses were given daily for 20 days.
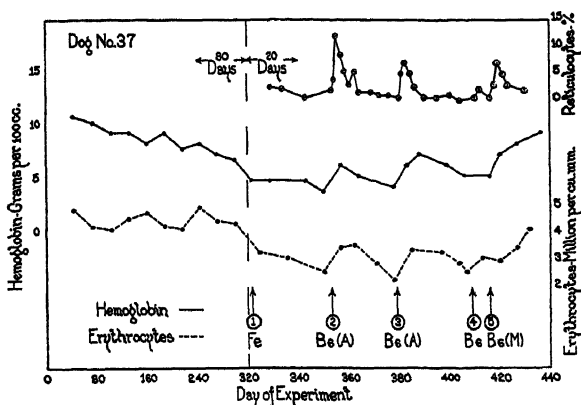
Fig. 3   Hemoglobin, erythrocyte and reticulocyte levels for dog 37. The first arrow indicates treatment with iron; 0.20 gm. of $FeSO_4 \cdot 7 H_2O$ were given daily for 17 days, followed by 0.5 gm. daily for 7 days. At two the authors' vitamin B_6 concentrate was given, 5 cc. per day for 4 days. At three the same concentrate was given, 40 cc. in 4 days. The fourth arrow indicates the administration of Lepkovsky's crystalline factor 1 (vitamin B_6), 4 mg. given as the first dose, 2 mg. on each of the 3 following days. This material was given subcutaneously dissolved in distilled water. At five the Merck vitamin B_6 concentrate was administered, twenty single rat doses daily for 13 days.

given orally, but the crystalline factor 1[13] was administered subcutaneously. It will be observed that both vitamin B_6 concentrates caused a rapid increase in hemoglobin and erythrocyte levels. Reticulocyte peaks were reached in from 1 to 3 days in the four cases in which the reticulocyte curves were followed. The failure of the crystalline factor 1 (vitamin B_6) to influence the anemia of dog 37 is surprising, particularly since the animal responded in a uniformly satisfactory manner to vitamin B_6 concentrates. The dose of crystalline factor 1 given was approximately 500 µg. per kilogram of body weight daily, whereas Fouts, Helmer and Lepkovsky ('39) have found that the dose of 60 µg. of crystalline factor 1 per kilogram, given orally, effected a remission of the anemia appearing in adult dogs on a diet lacking vitamin B_6. This same animal, dog 37, responded satisfactorily to B_6 concentrate[14] given in an amount furnishing approximately 400 µg. of vitamin B_6 per kilogram (point 5, fig. 3), calculated on the assumption that a single rat unit of vitamin B_6 is 0.10 mg. No quantitative assays were made on the authors' vitamin B_6 concentrate used in this study. However, one of us (H.R.S.) at a later date determined that a vitamin B_6 concentrate made by this procedure contains about one-fourth the potency of the original tikitiki, so that on this basis our B_6 concentrate contained about 10 µg. of B_6 per cubic centimeter. It is evident, therefore, that this crude concentrate produced a satisfactory hematopoietic response in doses supplying relatively small amounts of vitamin B_6.

Two possible explanations for the inactivity of crystalline factor 1 suggest themselves. It is conceivable that the subcutaneous route of injection, by which the crystalline material was administered, is less effective than oral administration. On the other hand, it is possible that after this long period on the experimental diet (409 days) the animal lacked more than one essential hematopoietic factor, and that the vitamin

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[13] We are indebted to Dr. Samuel Lepkovsky for a gift of his crystalline factor 1, or vitamin B_6 (Lepkovsky, '38).

[14] Merck's.

B$_6$ concentrate contained both or all of these, in contrast to the crystalline product.

It will be observed that large doses of ferrous sulfate given to dog 37 had absolutely no effect on the blood picture.

During the latter half of the experiment weekly leucocyte counts were made on all the animals. In contrast to the anemia observed in the animals of group 1, there was no tendency toward leucopenia, and no apparent difference between the leucocyte levels in the control group and those in the experimental group. Thus, in experimental dogs 31 and 33 the leucocyte counts varied from a minimum of 5100 to a maximum of 22,500 per cubic millimeter for the first animal, and from 5400 to 22,200 per cubic millimeter for the second. In the corresponding control animals, dogs 32 and 34, the leucocyte counts varied between 4300 and 14,900, and between 4100 and 7650, respectively.

Cardiac symptoms and pathology. Each of the animals in group 1 exhibited more or less marked symptoms of cardiac failure. These symptoms were most striking in dogs 31 and 33 and in both animals included dyspnea, tachycardia, large accumulations of serous fluid in the thorax, dilatation and hypertrophy of the right auricle and right ventricle, and chronic passive congestion of the liver. Dog 37 was more resistant to the shortcomings of the diet than the other animals in group 1, as indicated by the fact that it was the last to develop severe anemia; it never exhibited symptoms of cardiac embarrassment. However, when it was sacrificed on the 436th day there was found hypertrophy of both the right and left ventricles of the heart, more particularly of the right, and a slight dilatation of the right auricle. The heart was otherwise normal, as were the rest of the viscera. Sections of the liver showed a normal microscopic picture.

In contrast to the animals of group 1, the three controls of group 2 (dogs 32, 34 and 38) remained in excellent and vigorous health without the slightest suggestion of cardiac embarrassment. At autopsy their hearts looked entirely normal, as did the rest of the viscera.

The fourth control, dog 36, behaved anomalously throughout the experiment. As has already been mentioned, this animal served as an ad libitum control until the 348th day of the experiment when the vitamin B_6 concentrate was withdrawn in order to see whether symptoms of deficiency similar to those seen in the dogs of group 1 would appear. On the 369th day edema of the hind legs was observed, and 9 days later it was noticed that the abdomen was unusually full as though from ascites. By the 416th day distention of the abdomen had progressed to an alarming degree, and there was respiratory distress; the animal wheezed when placed on its back. When the dog was sacrificed on the following day, the thorax was found to be half full of clear serous fluid. Both the right ventricle and right auricle were greatly dilated and hypertrophied. The rest of the heart, including the valves, was normal. The abdomen was distended with several liters of clear fluid. There was marked edema everywhere; all the muscular tissues were jelly-like and dripping with fluid. The liver showed chronic passive congestion when examined histologically.

Peculiar attacks observed in dog 36. Shortly following the withdrawal of vitamin B_6 from the diet of this animal on the 348th day, the dog was observed to undergo periods of very transitory weakness and prostration. Six of these attacks were observed from the 352nd to the 361st day of the experiment, then no further were seen. In a typical attack, the animal would at first present a normal appearance, standing or walking in its cage, then suddenly it would begin to sway violently and then fall over. When picked up at this point it was completely limp and would make not the slightest effort to stand. About ¼ minute later the animal would get up and walk around in a normal manner as though nothing had happened. On one occasion the dog was observed to walk back and forth in its cage and jump up and down against the door in an excited manner 2 or 3 minutes before one of the attacks of weakness.

No seizures of this kind were ever seen in any of the other animals of this study. However, somewhat similar symptoms have been reported in pigs suffering from lack of vitamin B_6.

Chick and co-workers ('38) stated that five pigs on a diet lacking vitamin B$_6$ occasionally exhibited epileptic fits. According to their description, in these seizures the animal at first ran about wildly, then fell over as though shot, and became pale and comatose for a few minutes. Recovery was rapid, and the animal would resume its previous occupation. The attacks of weakness occurring in dog 36 are thus similar to those seen in pigs on a similar diet, if one assumes that the excitement phase is less pronounced in the dog.

TABLE 1

Myelin degeneration appearing on a diet lacking vitamin B$_6$

	LENGTH OF EXPERIMENTAL PERIOD	SCIATIC NERVE		SPINAL CORD		
		Marchi	Spiel-meyer	Marchi	Spiel-meyer	Nissl
	days					
Group 1						
Experimental						
Dog 31	355	4+	2+	3+	+	slight gliosis
Dog 33	317	2+	0	+	0	0
Dog 37	436	0	0	0	0	0
Dog 43	199	2+	0	0	0	0
Group 2						
Controls						
Dog 32	433	2+	0	0	0	0
Dog 34	319	3+	3+	0	0	0
Dog 38	319	2+	0	0	0	0
Group 3						
Dog 36	417	2+	+	±	0	0

Histopathology of the nervous system. Microscopic examination of the nervous system revealed degenerative changes in the myelin sheaths of certain of the animals; these are detailed in table 1. It will be observed that both experimental and control groups showed moderate to fairly severe degeneration of sciatic nerves. However, none of the three control animals exhibited any changes in the spinal cord, while two of the dogs in group 1 had myelin degeneration in the posterior columns and dog 36 showed slight degeneration of the posterior columns. There was no high degree of correlation

between the clinical symptoms of deranged neuromuscular function and the histologic findings. It is true that dog 31, with the most striking clinical symptoms showed the most severe myelin destruction, but on the other hand, the nervous system of dog 37 was essentially intact in microscopic appearance, yet this animal had developed a slight ataxia before the end of the experiment. This animal was the last to develop the characteristic symptoms of vitamin B_6 deficiency, presumably because of protection from vitamin deficiency through coprophagy. It was observed to eat its feces until it was muzzled in the latter part of the experiment.

The results of this study do not settle with any finality the role of vitamin B_6 in the functioning of the nervous system, since we did not succeed in entirely preventing myelin changes in the control group receiving vitamin B_6. However, we feel that the most probable explanation of our observations is that the amount of vitamin B_6 given to the controls (the equivalent of 1.5 gm. of rice polish per kilogram daily) while adequate to maintain them in apparently good health, was, after all, not quite enough to prevent early changes in the nervous system. This seems all the more probable, since this amount of vitamin B_6 was definitely too small to maintain health in dog 36.

Gastric juice analyses. Between the 265th and 342nd day of the experiment, several analyses of gastric juice were made on the seven surviving dogs. Following an overnight fast, 25 cc. of 7% alcohol were administered, and the stomach contents were removed as completely as possible 30 minutes later through a Reyfuss tube. The results are summarized in table 2. It will be observed that the volumes of the samples obtained from experimental dogs 31 and 33 were much smaller than those from the three control animals; also the average titer of free acid was about half that of the controls. Occasionally, samples were obtained from dogs 31 and 33 which contained no free acid. However, there was never a true achlorhydria, since stimulation with histamine in these two dogs promoted the secretion of juice containing normal amounts of free acid. In one of the animals there was an indication that the admin-

istration of vitamin B$_6$ concentrate might act to increase the free acid content of the gastric juice; in the case of dog 33, three determinations of free acid just prior to treatment with vitamin B$_6$ gave values of 9, 3 and 19, expressed as cubic centimeters of N/10 HCl per 100 cc. of gastric juice. Following treatment with the B$_6$ concentrate[15] (fig. 2) the next four determinations gave values of 37, 0, 48 and 58 cc. of free acid.

TABLE 2

Analyses of gastric juice samples obtained after stimulation with a 7% alcohol test meal

GROUP	DOG NO.	NUMBER OF DETERMINATIONS	AVERAGE VOLUME OF SAMPLES	AVERAGE VALUE FOR FREE ACID
			cc.	*cc. of N/10 acid per 100 cc.*
Experimental	31	5	7.2	26
	33	7	6.9	25
	37	6	15.7	45
Control	32	2	21.5	49
	34	2	15.0	47
	38	3	29.3	57

DISCUSSION

Our results confirm the findings of Fouts et al. ('38), using dogs, and Chick and associates ('38), using pigs, that a diet containing thiamine, riboflavin and filtrate factor, but not vitamin B$_6$, leads to the development of a microcytic anemia. Our inability to effect a cure of the anemia with crystalline vitamin B$_6$ would seem to suggest that other factors may be involved in the production of anemia with the particular diet that we have used. Further work is needed to clarify this point.

We believe that the finding in several of our animals of well-advanced dilatation and hypertrophy of the right side of the heart, with other symptoms of congestive heart failure, is a matter of some interest since, to our knowledge, such a condition has not been produced in nutrition studies except in vitamin B$_1$ deficiency. Lack of vitamin B$_1$ could hardly be a factor in the present study, since the animals received 10 µg. of vitamin B$_1$ per kilogram daily, whereas we have found

[15] See footnote 14, p. 282.

(Street, Zimmerman, Cowgill, Hoff and Fox, '41) that the daily administration of 7 to 9 µg. of vitamin B_1 per kilogram prevents the cardiac hypertrophy which occurs in dogs subsisting on a diet lacking vitamin B_1. The present study does not show whether or not the congestive heart failure was due specifically to lack of vitamin B_6, but since the condition was prevented by the administration of a vitamin B_6 concentrate, it would appear that the cardiac dysfunction was presumably caused either by lack of vitamin B_6 or some as yet unidentified factor occurring in B_6 concentrates. It is interesting to note that in dog 36, which suffered from only a moderate shortage of vitamin B_6, marked symptoms of cardiac embarrassment appeared at the close of the experiment, despite the fact that the appetite was good throughout most of the experimental period, and there was only moderate loss of weight (13%), while the behavior was essentially that of a normal animal. This would suggest that lack of a dietary essential may sometimes cause cardiac derangement in the absence of other obvious symptoms of inadequate nutrition.

Likewise, in the case of the myelin degeneration of the nervous system it is not possible to state that lack of vitamin B_6 is the etiological factor; it is clear, however, that some factor in the vitamin B_6 concentrate given the control dogs considerably reduced the severity of the pathological changes in the nervous system. With the exception of vitamin B_1, the role of the individual members of the vitamin B complex in preserving the integrity of the nervous system is largely unexplored. However, Wintrobe, Mitchell and Kolb ('38) have demonstrated that lack of members of the B group other than B_1 and riboflavin may cause myelin degeneration in pigs, and the present study indicates that vitamin B_6 may be one of the further factors essential for the maintenance of the nervous system.

The moderately severe myelin degeneration of the peripheral nerves observed in the three control dogs of group 2 after the conclusion of the study was surprising to us, since during the experiment these animals were lively, had good appetites and, in general, appeared to be in excellent health. This obser-

vation suggests that a dietary regime which maintains apparent health is not necessarily adequate for the preservation of the integrity of the nervous system over extended periods.

It is clear that there is a great difference in the form which vitamin B_6 deficiency takes in the dog as compared to the rat. Although severe dermatitis of the paws and nose is the chief symptom of this condition in the rat, in the adult dogs used in this study, there were no cutaneous symptoms whatever. There is also a striking difference between the picture of chronic disease that we have produced by lack of vitamin B_6 in adult dogs, and the very rapidly developing deficiency state which McKibben, Madden, Black and Elvehjem ('39) observed in puppies given a diet lacking vitamin B_6. In these puppies no definite, consistent deficiency symptoms were seen other than anemia, loss of weight, anorexia and death. Undoubtedly puppies are superior as assay animals in studying the preparation of vitamin B factors needed by the dog but, on the other hand, adult dogs are superior for studying the nature of the syndromes which one might expect to appear in man as a result of lack of the various B factors.

SUMMARY

Dogs maintained on a diet containing, of the B vitamins, thiamine, riboflavin, and filtrate factor, but not vitamin B_6, developed a severe anemia within 120 to 320 days. Treatment with vitamin B_6 concentrates led to a rapid increase of erythrocyte and hemoglobin levels, while treatment with ferrous sulfate produced no response.

After 300 days or more on the diet some of the animals developed marked symptoms of cardiac embarrassment, including dyspnea, tachycardia, dilatation and hypertrophy of the right ventricle and right auricle, accumulation of serous fluid in the thorax, and chronic passive congestion of the liver.

Degenerative changes were found in the myelin sheaths of the peripheral nerves and of the spinal cords of the experimental animals. These changes were of lighter degree in the control animals, receiving vitamin B_6 concentrate, and occurred only in the peripheral nerves.

Analyses of the gastric juice carried out in the latter part of the experiment gave a suggestion that in vitamin B_6 deficiency there may be a decrease in the secretion of acid and possibly in the volume of juice secreted.

Control animals receiving the same diet, except that they were given vitamin B_6 concentrate in addition, did not become anemic and showed no evidence of cardiac dysfunction either during life or at autopsy.

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CHOLINE METABOLISM

V. THE EFFECT OF SUPPLEMENTARY CHOLINE, METHIONINE AND CYSTINE AND OF CASEIN, LACTALBUMIN, FIBRIN, EDESTIN AND GELATIN IN HEMORRHAGIC DEGENERATION IN YOUNG RATS [1]

WENDELL H. GRIFFITH

Department of Biological Chemistry, St. Louis University School of Medicine, Missouri

THREE FIGURES

This investigation is a continuation of previously reported observations of hemorrhagic degeneration in young rats (Griffith and Wade, '39, '40; Griffith, '40 a and '40 b) and is a study of the influence of methionine and of cystine on the requirement of choline. The occurrence and severity of hemorrhagic degeneration have been determined in rats fed twenty-five diets varying in protein content from 18 to 40%, in total methionine from 0.4 to 1.2% and in total cystine from 0.05 to 1.2%. The proteins, casein, lactalbumin, fibrin, edestin and gelatin were used with and without supplements of methionine and cystine. Variations in the degree of choline deficiency were measured by the severity of the characteristic renal hemorrhage, by the increase in the weight of the kidneys and by the extent of deposition of fat in the liver.

EXPERIMENTAL

The experimental procedures and the constituents of the diet were similar to those previously described (Griffith and Wade, '39). The appearance of the kidneys was noted at the

end of the experimental period and the per cent of animals in each group showing the hemorrhagic state (Griffith, '40 b) is recorded in tables 1 to 4 and in figure 2 under the heading "renal lesions." Determinations were made of liver and kidney weights and of the total chloroform-soluble substances in the liver. The term "liver fat" in tables 2 to 4 and in figure 3 refers to this fraction.

Young male rats, 20 days of age and 25 to 34 gm. in weight, were used in the experiments recorded in table 1. The basal diet, AC 34, consisted of purified casein 15%, lard 9, sucrose 62.9, salt mixture 4 (Hawk and Oser, '31), calcium carbonate 1, agar 2, whole dried yeast 6, and the fortified fish liver oil,[2] 0.1%.

In the experiments listed in tables 2 to 4 young male rats, 21 to 26 days of age and 38 to 42 gm. in weight were employed. The basal diet was the same as AC 34 except that lard was increased to 20% and various protein levels and mixtures were used. All additions to the basal diet replaced an equal weight of sucrose. The composition of the protein fraction of each ration is indicated in tables 2 to 4. These tables also show the total methionine and cystine in each diet, exclusive of the yeast protein. The values for these sulphur-containing amino acids in casein, lactalbumin, fibrin, edestin and gelatin were taken from the data of Baernstein ('32, '34 and '36) and are as follows: methionine, 3.1, 2.3, 2.4, 2.2, 1.0% respectively and cystine, 0.3, 3.4, 1.7, 1.4, 0.5% respectively. Supplements of l-cystine and of dl-methionine were used in certain diets. Best and Ridout ('40) have found the racemic mixture as potent as the natural methionine in the prevention of fatty livers in older rats.

<center>RESULTS</center>

Relation of dietary cystine to hemorrhagic degeneration. Table 1 shows the occurrence of renal lesions and figure 1 the corresponding increase in kidney weight on diets containing 0, 0.3, 0.5 and 1.0% of added cystine. In addition, the intake

[2] Natola.

TABLE 1

Effect of choline and of cystine on hemorrhagic degeneration in young male rats [1]

GROUP	NUMBER OF RATS	FINAL BODY WEIGHT	FOOD PER DAY	CHOLINE CHLORIDE ADDED PER GRAM OF FOOD	CYSTINE ADDED PER GRAM OF FOOD	PER CENT OF RATS WITH RENAL LESIONS
		gm.	gm.	mg.	mg.	
A 1	40	43	4.6	0	0	80
A 2	40	45	5.0	0.125	0	30
A 3	25	46	5.4	0.250	0	4
B 4	39	40	3.4	0	3	92
B 5	49	45	4.4	0.125	3	76
B 6	36	47	4.9	0.250	3	45
B 7	22	52	5.3	0.375	3	0
C 8	30	42	4.0	0	5	93
C 9	40	47	4.5	0.125	5	80
C 10	20	49	4.7	0.250	5	70
C 11	17	49	4.8	0.375	5	0
C 12	12	52	5.2	0.50	5	0
D 13	30	40	3.9	0	10	100
D 14	46	44	4.1	0.125	10	83
D 15	34	48	4.7	0.250	10	41
D 16	25	49	5.0	0.500	10	0

[1] Experimental period—7 days. Average weight at start—29 gm. Age at start—20 days. Basal diet AC 34.
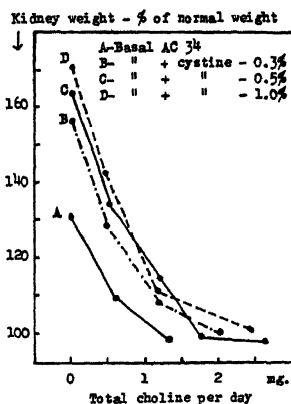
Fig. 1 The effect of choline and of cystine on the increase in kidney weight due to renal hemorrhagic degeneration. The occurrence of renal lesions in these groups is recorded in table 1.

of choline necessary to maintain the normal appearance and
weight of the kidneys was determined for each level of added
cystine. Although these results confirmed the previously re-
ported injurious effect of cystine (Griffith and Wade, '40),
it was evident that renal damage was not at all proportional
to the amount of cystine ingested. The minimum daily re-
quirement of choline for protection of the kidneys was the
same regardless of whether the diet contained 0.3 or 1.0%
of added cystine. However, the higher cystine levels were
slightly more toxic in the absence of added choline. It would
appear either that only a certain fraction of the dietary
cystine was metabolized in such a way that extra choline was
required or that variable quantities of cystine were normally
metabolized in the presence of a fixed amount of choline. In
these experiments cystine was not found to be a toxic amino
acid if the diet contained an adequate supply of choline.

The effect of various proteins on hemorrhagic degeneration.
Table 2 shows the relative effects of a low choline diet contain-
ing 18% of casein and of diets containing 12% of casein plus
6% of lactalbumin, fibrin, edestin or gelatin (groups 1 to 5).
Substitution of part of the casein with these proteins did not
prevent the effects of choline deficiency since, in each instance,
deposition of liver fat was excessive and renal lesions were
severe. These five diets were then compared after the addi-
tion of choline (groups 6 to 10, table 2) inasmuch as it has
been our experience that significant differences which were
masked on severe diets, might appear if the diets were supple-
mented with partially protective levels of choline. It was
clearly evident from the results that casein was the most
protective of the proteins used and that the effects of choline
deficiency were aggravated by lactalbumin, fibrin, edestin and
gelatin. The inclusion of these four proteins in diets produced
food mixtures containing less methionine and more cystine, a
shift which increased the requirement of choline (Griffith and
Wade, '40).

*The relation between the methionine content of the diet and
hemorrhagic degeneration.* Tables 3 and 4 show the varying

TABLE 2

The effect of dietary proteins on the deposition of liver fat and on the occurrence and severity of hemorrhagic degeneration in 40-gm. male rats after an 8-day experimental period

GROUP [1]	1	2	3	4	5	6	7	8	9	10
Choline chloride added per gram of food, mg.	0	0	0	0	0	0.2	0.2	0.2	0.2	0.2
Dietary protein										
casein, %	18	12	12	12	12	18	12	12	12	12
Lactalbumin, %	0	6	0	0	0	0	6	0	0	0
Fibrin, %	0	0	6	0	0	0	0	6	0	0
Edestin, %	0	0	0	6	0	0	0	0	6	0
Gelatin, %	0	0	0	0	6	0	0	0	0	6
Average final body weight, gm.	60	54	58	56	54	65	63	61	62	62
Average food consumption per day, gm.	5.1	4.4	4.6	4.4	4.6	5.5	5.1	4.9	5.3	5.4
Liver weight as per cent of body weight [2]	6.9	7.4	6.7	6.7	6.6	5.8	6.8	6.5	6.4	6.6
Average weight of liver fat, mg.	897	1009	990	895	895	536	1030	872	828	870
Liver fat as per cent of liver [3]	21.5	25.0	25.2	23.7	25.3	14.1	23.9	21.7	21.1	21.4
Average weight of kidneys (per pair), mg.	928	1002	954	955	935	731	790	811	772	811
Kidney weight as per cent of body weight [4]	1.54	1.85	1.63	1.71	1.73	1.13	1.25	1.32	1.25	1.31
Renal hemorrhage, %	81	86	86	95	86	0	48	48	52	43
Total methionine per gram of food, mg.	5.6	5.1	5.2	5.0	4.3	5.6	5.1	5.2	5.0	4.3
Total cystine per gram of food, mg.	0.5	2.4	1.4	1.2	0.7	0.5	2.4	1.4	1.2	0.7

[1] Each group consisted of 21 rats, 21 to 26 days of age and 38 to 42 gm. in weight.
[2] Normal per cent of body weight for rats in this colony, 4.31.
[3] Normal per cent of liver for rats in this colony, 4.0.
[4] Normal per cent of body weight for rats in this colony, 1.0 to 1.2.

TABLE 3

The effect of dietary proteins and of added methionine and cystine on the deposition of liver fat and on the occurrence and severity of hemorrhagic degeneration in 40 gm. male rats after an 8-day experimental period

GROUP [1]	11	12	13	14	15	16	17	18	19	20 [2]	21 [3]
Dietary protein											
Casein, %	12	0	18	18	40	40	18	18	18	18	18
Lactalbumin, %	0	16	22	0	0	0	0	0	0	0	0
Fibrin, %	12	16	0	22	0	0	0	0	0	0	0
Gelatin, %	0	0	0	0	0	0	22	22	22	22	22
Methionine added per gram of food, mg.	0	0	0	0	0	0	0	0	4	0	0
Cystine added per gram of food, mg.	0	0	3	3	3	10	3	0	10	10	0
Average final body weight, gm.	62	59	63	63	67	64	58	56	63	59	60
Average food consumption per day, gm.	5.4	5.0	5.3	5.1	5.2	4.7	4.7	4.2	4.7	4.5	4.5
Liver weight as per cent of body weight [4]	6.3	5.5	5.1	5.0	5.2	5.5	6.5	6.2	5.3	5.1	4.8
Average weight of liver fat, mg.	738	432	140	149	196	166	845	723	273	370	134
Liver fat as per cent of liver [5]	19.0	13.4	4.3	4.8	5.6	4.7	22.5	20.9	8.2	12.3	4.7
Average weight of kidneys (per pair), mg.	835	758	812	765	815	880	1080	1061	1070	1025	975
Kidney weight as per cent of body weight [6]	1.34	1.28	1.29	1.21	1.22	1.37	1.86	1.92	1.71	1.73	1.63
Renal hemorrhage, %	29	10	0	0	0	14	81	50	10	10	10
Total methionine per gram of food, mg.	6.6	7.5	10.6	10.9	12.4	12.4	7.8	7.8	11.8	7.8	7.8
Total cystine per gram of food, mg.	2.4	8.2	11.0	7.3	4.2	11.2	4.6	1.6	11.6	11.6	1.6

[1] Each group consisted of 21 rats, 21 to 26 days of age and 38 to 42 gm. in weight.
[2] 0.5 mg. of choline chloride added per gram of food.
[3] 2.0 mg. of choline chloride added per gram of food.
[4] Normal per cent of body weight for rats in this colony, 4.31.
[5] Normal per cent of liver for rats in this colony, 4.0.
[6] Normal per cent of body weight for rats in this colony, 1.0 to 1.2.

TABLE 4

The effect of dietary proteins and of added methionine and cystine on the deposition of liver fat and on the occurrence and severity of hemorrhagic degeneration in 40-gm. male rats after an 8-day experimental period

GROUP [1]	22	23	24	25	26	27	28	29	30	31	32
Dietary protein											
Casein, %	18	18	18	18	18	9	9	9	9	9	0
Lactalbumin, %	0	0	0	0	0	3	3	3	3	3	9
Fibrin, %	0	0	0	0	0	3	3	3	3	3	9
Gelatin, %	0	0	0	0	0	3	3	3	3	3	0
Methionine added per gram of food, mg.	1	0	0	1	3	0	1	2	4	6	0
Cystine added per gram of food, mg.	0	1	3	3	3	3	3	3	3	3	0
Average final body weight, gm.	65	57	59	61	67	56	58	66	66	67	54
Average food consumption per day, gm.	5.4	4.6	4.6	4.8	5.5	4.4	4.6	5.3	5.4	5.3	....
Liver weight as per cent of body weight [2]	6.4	6.9	7.4	6.4	5.3	7.6	7.1	6.3	5.2	5.3	7.0
Average weight of liver fat, mg.	721	861	1115	885	284	1097	1142	973	274	201	1103
Liver fat as per cent of liver [3]	17.3	22.0	25.5	22.8	8.0	26.2	28.0	23.7	8.0	5.7	29.2
Average weight of kidneys (per pair), mg.	759	1028	1036	840	725	980	973	828	756	792	895
Kidney weight as per cent of body weight [4]	1.16	1.82	1.75	1.38	1.08	1.77	1.68	1.26	1.15	1.19	1.66
Renal hemorrhage, %	29	86	95	67	0	95	86	43	0	0	91
Total methionine per gram of food, mg.	6.6	5.6	5.6	6.6	8.6	4.5	5.5	6.5	8.5	· 10.5	4.2
Total cystine per gram of food, mg.	0.5	1.5	3.5	3.5	3.5	5.0	5.0	5.0	5.0	5.0	4.6

[1] Each group consisted of 21 rats, 21 to 26 days of age and 38 to 42 gm. in weight.
[2] Normal per cent of body weight for rats in this colony, 4.31.
[3] Normal per cent of liver for rats in this colony, 4.0.
[4] Normal per cent of body weight for rats in this colony, 1.0 to 1.2.

degrees of protection against choline deficiency on diets differ-
ing in total protein, methionine and cystine. It was previ-
ously shown by Griffith and Wade ('40) that a 40% level
of casein in a ration was completely protective whereas 15
and 25% levels were injurious. The same defense against
hemorrhagic degeneration was apparent on food mixtures
containing 18% casein plus 22% lactalbumin or fibrin even
though extra cystine was added (groups 13 to 15, table 3).
Similar protection resulted from the addition of methionine
to diets containing 18% protein (groups 26, 30 and 31, table 4).
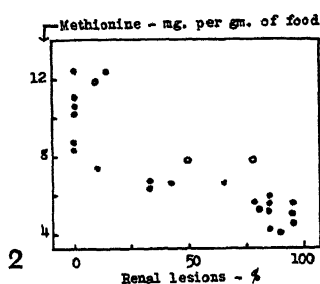
Fig. 2   The relation between the methionine content of the diet and the
occurrence of renal lesions in the twenty-five groups of rats in tables 2, 3 and 4
which did not receive added choline. Diets containing 22% of gelatin are repre-
sented by circles.
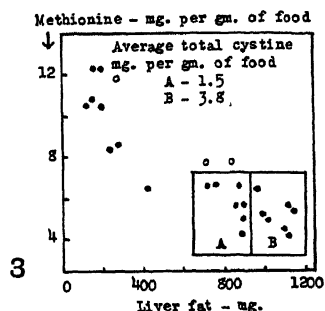
Fig. 3   The relation between the methionine content of the diet and the deposition
of liver fat in the twenty-five groups of rats in tables 2, 3 and 4 which did not
receive added choline. Diets containing 22% of gelatin are represented by circles.

The fact that hemorrhagic degeneration was absent, or nearly
so, in each group receiving more than 8 mg. of methionine per
gram of food, indicated that dietary choline was not essential
if the food mixture supplied sufficient methionine. This effect
of methionine is clearly evident in figures 2 and 3 in which
total methionine was plotted against renal lesions and liver
fat, respectively, for the twenty-five different diets listed in
tables 2, 3 and 4 which did not contain added choline. Choline
appeared to be a dietary essential in these diets only if less
than 8 mg. of methionine per gram of food was present. With
less than 8 mg. the renal lesions and deposition of liver fat

occurred and varied according to the methionine and cystine levels. Group 16, table 3, was the only high methionine group exclusive of the high gelatin diets (groups 17 to 21, table 3) which failed to show complete protection against hemorrhagic degeneration. Only three of the twenty-one rats showed renal lesions and the livers were not fatty. It has been exceptional for renal hemorrhage to occur in the absence of a deposition of liver fat.

Anomalous results followed the feeding of diets containing 18% of casein and 22% of gelatin (groups 17 to 21, table 3). Although groups 17, 18 and 19 (shown by circles in figures 2 and 3) did not deviate greatly from the other groups and although the addition of extra methionine (group 19) and of choline (groups 20 and 21) decreased the deposition of liver fat and the occurrence of renal lesions, there was in every case a marked increase in the weight of the kidneys. Such an enlargement of the kidneys without the simultaneous occurrence of hemorrhagic degeneration has been observed in this investigation only on diets containing a large amount of gelatin.[3] The fact that these food mixtures also contained 18% of casein indicated that the effect of gelatin was positive in its nature. This finding confirmed the similar observation of Jackson, Sommer and Rose ('28) who noted severe renal pathology in rats fed a ration high in gelatin.

DISCUSSION

Our investigation of choline metabolism in young growing rats has been concerned primarily with the relation of choline to the maintenance of normal renal structures and only secondarily to the relation between choline and liver fat. The investigations of others (Best and Ridout, '39) on the production and prevention of fatty livers have utilized rats 100 to 200 gm. in weight in which hemorrhagic degeneration has not been reported. Furthermore, in such experiments it has been customary to use high fat (40%) and very low protein (5%)

[3] More recent observations indicate that these kidneys may have been hemorrhagic very early in the experimental period and were actually in the "recovered" stage.

rations of a type unsuitable for young rats. The results obtained by these two experimental procedures have been characterized for the most part by their uniformity rather than by differences although there is as yet no certainty that the many factors which control the relation of choline to hemorrhagic degeneration in young rats are the same as those which affect liver fat in older animals. In this connection it is of interest that Tucker, Treadwell and Eckstein ('40) have recently reported that the lipotropic action of a diet containing 20% casein could be duplicated by the proper supplements of methionine and cystine in a diet containing 5% casein. Also Channon, Manifold and Platt ('40) have found that the daily intake of more than 4 mg. of cystine exerted no additional effect in promoting the deposition of liver fat in older rats.

In the present study supplementary methionine or protein methionine prevented hemorrhagic degeneration and, therefore, satisfactorily replaced choline in the diet of young rats. This result confirmed the suggestion of Griffith and Wade ('40) that protection on high casein diets may have been due to the high methionine content of such diets and supported the conclusion of du Vigneaud, Chandler, Moyer and Keppel ('39) that methionine may be a precursor of choline and may serve as a source of required methyl groups. Under the conditions of this investigation, diets containing 0.8% of methionine supplied sufficient amounts of this amino-acid for the synthesis of choline and for other indispensable functions. In the absence of added choline, hemorrhagic degeneration occurred if the diet contained less than 0.8% of methionine. It was not possible to state exactly the daily choline requirement for young rats except for specified food mixtures since the amount depended upon dietary methionine and other components of the ration, as well as upon the age and sex of the experimental animals (Griffith, '40 b). The minimum requirement of methionine which in these experiments was 0.8% of the diet and which Rose ('37) found to be 0.6% of the diet (for normal growth of young rats) should vary with the

ration and should depend in part upon the level of dietary choline.

In view of the probability that methionine may replace choline it might seem inadvisable to consider the latter a dietary essential. However, choline should be included in the list of indispensable nutrients for the young rat because the high level of methionine required in the absence of choline may be obtained only by the use of unnecessarily high protein levels or by the addition to the diet of methionine itself. Unpublished data indicate that such supplementary methionine, while effective, is less efficient than the theoretical equivalent of choline (on the basis of methyl groups). Furthermore, the fact that a diet containing as much as 25% of casein, one of the most protective high-methionine and low cystine proteins, required supplementary choline emphasized the necessity of additions of this substance to rations fed young rats in nutritional investigations.

The role of cystine in intensifying hemorrhagic degeneration remains unexplained. If cystine may be formed from methionine, then supplementary cystine should spare methionine so that more of the latter might be available for synthesis of choline, i.e., less choline should be needed in the diet. Actually, the reverse was true, more choline being required. This experimental result would be explicable if the metabolism of cystine directly involved that of choline. Or, if methionine were required for the metabolism of cystine, then supplementary cystine in low choline-low methionine diets should use up methionine so that less of the latter would be available for choline formation. In this connection the observation that the effect of cystine in increasing the requirement of choline was not proportional to the level of added cystine may be of considerable importance. Since 1.0% of added cystine required no more choline than 0.3% for prevention of hemorrhagic degeneration, then 0.7% of added cystine appeared to be used in such a way that choline was not involved, whereas 0.3% (or some smaller amount) required the simultaneous metabolism of choline. The rate of absorption from the

gastrointestinal tract was probably not a limiting factor in these experiments in which supplementary cystine (15 to 50 mg. per day) was consumed with the food inasmuch as the rate of absorption of this amino acid in rats has been found to be approximately 30 mg. per hour per 100 gm. of body weight (Wilson, '30).

Womack, Kemmerer and Rose ('37) demonstrated that cystine was dispensable in a ration which was an adequate source of methionine. However, Rose ('37) noted that a supplement of cystine greatly improved the quality of the diet for growth if methionine was supplied in insufficient amounts. Such a conception of the value of dietary cystine is involved in the following tentative explanation of the injurious effect of cystine which was observed on diets low in choline, in methionine and in cystine. On such diets there was definitely too little methionine to satisfy the demand for choline and possibly also for cystine so that the metabolism of the rat may have been limited by a double deficiency. A supplement of cystine, instead of sparing methionine, might have increased the need for methionine and choline by improving the state of nutrition. In other words the alleviation of the cystine deficiency might have aggravated the choline deficiency because of increased metabolism or because of a closer approach to normal metabolism. It has been apparent from the start of this investigation that signs of choline deficiency were most acute on low choline, low methionine diets which were superior in other respects (Griffith and Wade, '39). The proposed role of cystine obviates the necessity of assuming a direct connection between the metabolism of cystine on the one hand and that of methionine and choline on the other and also obviates the necessity of postulating a direct relation between choline and a part, but not all, of the cystine. This hypothesis, which is being investigated further, would explain the similarity in results following the addition to diets of 0.3 and 1.0% cystine since the effective level would depend only upon the need for cystine.

The importance of metabolites which may serve as sources of labile and essential methyl groups is indicated in numerous current investigations dealing with apparently divergent phases of metabolism. In this respect methionine is of unusual significance in the body not only as an amino acid but also as an intermediary which appears to be related to choline, to the turnover of phospholipids (Perlman, Stillman and Chaikoff, '40), to betaines (Chandler and du Vigneaud, '40) and to creatine (du Vigneaud, Chandler, Cohn and Brown, '40; Borsook and Dubnoff, '40). In addition methionine may play a role in the detoxication of selenium (Lewis, Schultz and Gortner, '40) and of other toxic compounds (Stevenson and White, '40). Hydrogen sulphide formed by micro-organisms in the intestinal tract (Wohlgemuth, '05) may also require methylation. It is of interest that dimethyl sulphone has been identified as a tissue constituent (Ruzicka, Goldberg and Meister, '40; Pfiffner and North, '40). The prevention of hemorrhagic degeneration in young rats is a relatively sensitive test for those metabolites which may supply methyl groups for the synthesis of choline. This aspect of the general problem of biological methylations and demethylations is being continued.

SUMMARY

1. Hemorrhagic degeneration in young rats is absent or slight if a low choline diet contains more than 0.8% of methionine, regardless of whether this level is attained by supplementary methionine or by protein levels high in casein, lactalbumin or fibrin. These results support the conclusion that methionine supplies methyl groups for the synthesis of choline.

2. Choline is a dietary essential if the ration contains less than 0.8% of methionine.

3. The choline requirement varies inversely with the dietary methionine and is increased by dietary cystine.

4. The effect of cystine is observed only on diets low in cystine and low in either choline or methionine and is not proportional to the level of supplementary cystine. A possible explanation of the effect of cystine is proposed.

5. An exceptional increase in kidney weight results on diets containing 18% of casein and 22% of gelatin even though hemorrhagic degeneration is prevented by methionine or by choline.

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THE EFFECT OF VARIOUS AMOUNTS OF CAFFEINE ON THE GASEOUS EXCHANGE AND THE RESPIRATORY QUOTIENT IN MAN

JOHN HALDI, GEORGE BACHMANN, CHARLES ENSOR AND
WINFREY WYNN

T. T. Fishburne Laboratory of Physiology, Emory University, Georgia

TWO FIGURES

Numerous investigations have been made on the effect of caffeine on various physiological processes but only a few have been concerned with the effect of the drug on the respiratory exchange including the respiratory quotient in man. Studies in which caffeine was administered in the form of coffee or tea may be excluded from consideration because of the extraneous factors that were necessarily involved. Edsall and Means ('14), Higgins and Means ('15), and Means, Aub and DuBois ('17) found that caffeine in relatively large doses stimulates metabolism but has no effect on the respiratory quotient. In each of these investigations the conclusions reached were based on a very small number of experiments. In the first there was only one experiment on each of two subjects in which, respectively, 0.390 and 0.324 gm. caffeine salicylate was injected subcutaneously; in the second, the data on the respiratory exchange are presented for only one experiment on each of two subjects in which caffeine sodium benzoate or caffeine sodium salicylate was injected in the amounts of 0.32, 0.40 and 0.50 gm.; in the third there was a total of five experiments on four subjects in which approximately 8.6 mg. per kilo body weight was administered by mouth in 300 cc. water. In view of the small number of experiments so far reported it was deemed advisable to reinvestigate the problem.

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METHOD

The subjects of these experiments were two male adults, one of whom (W.W.) weighed 60 and the other (C.E.) 66 kg. Both used coffee regularly and in moderation but abstained from all beverages containing caffeine during the 24 hours preceding an experiment. The subject came to the laboratory in the fasting state at about 7 : 30 in the morning, having taken no food after 7 o'clock the preceding evening. He then reclined for 30 to 45 minutes before the experiment was begun.

The respiratory exchange was determined by the open circuit method of Carpenter and Fox ('31) with a slight modification as described elsewhere (Bachmann and Haldi, '37). Aliquot samples of expired air collected over consecutive 15-minute periods were analyzed for oxygen and carbon dioxide with the Haldane-Henderson analyzer. Check determinations were made on each sample by two analysts.

After the preliminary rest the gaseous exchange was determined for three basal periods. The caffeine dissolved in 200 cc. water at 37°C., or the same amount of water alone in the control experiments, was then ingested. The determination of gaseous exchange was resumed immediately and continued for seven periods (1¾ hours). The recumbent position was maintained throughout the experiment except for a period of 3 or 4 minutes when the subject sat up to ingest the water or solution. Three different dosages of caffeine were used, namely: 1, 3 and 6 mg. per kilo body weight. Five experiments with each dosage and the same number of controls were done on each subject, or a total of forty experiments. Care was taken that the experiments with caffeine were so spaced that there would be no after-effect of the preceding experiment.

RESULTS

The rise of the respiratory quotient above the average basal for the seven 15-minute post-ingestion periods, together with the increase in the oxygen consumption and carbon dioxide elimination are shown in graphic form in figure 1. Each point

on the curves represents an average obtained from five experiments.

Respiratory quotient. Six milligrams caffeine per kilo produced a distinct rise in the respiratory quotient. In one subject the rise reached its peak the first 15-minute period and in
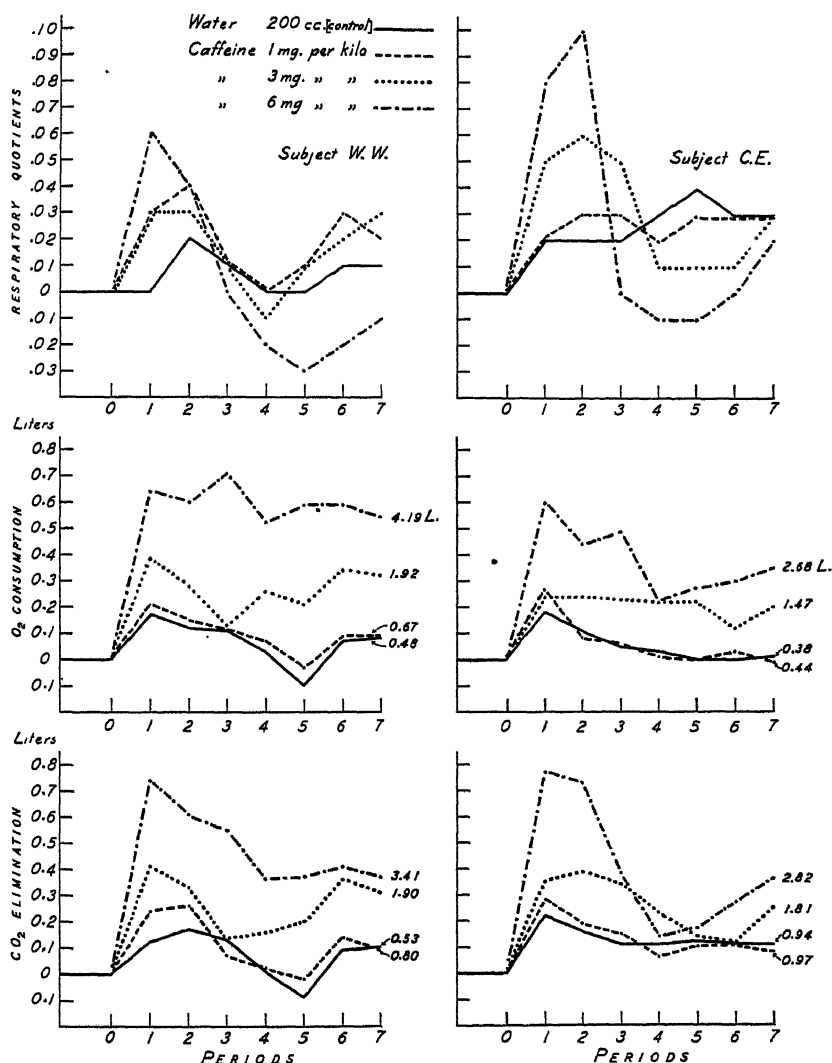
Fig. 1   The course of respiratory metabolism with reference to basal conditions and control experiments, following the administration of various doses of caffeine.

the other the second period after ingestion. Immediately after attaining its highest level the quotient fell and in the fifth period was well below the base line. In the seventh period at the conclusion of the experiment it had returned practically to the basal level.

The 3 mg. per kilo dose also was followed by an elevation of the respiratory quotient which, however, was not as pronounced as with the larger dose. The quotient followed the same general course as when 6 mg. caffeine per kilogram was taken. It is of interest to note that with the lesser rise in the quotient following the smaller dosage there was subsequently a lesser dip below the base line.

One milligram caffeine per kilo had no effect on the quotient of C.E. whereas it produced a rise practically the same as that produced by 3 mg. per kilo in the quotient of W.W. Apparently the two subjects had a different threshold of sensitivity to the drug.

Oxygen consumption. Metabolism was definitely stimulated by the two larger doses of caffeine as shown by the rise in the oxygen consumption. The stimulating effects were still evident at the end of the seventh period when the experiment was concluded. The figures on the right-hand side of the graphs give the number of liters of oxygen consumed in excess of basal requirements, on the assumption that the basal level determined for three periods preceding ingestion remained the same throughout the experiment. The average increase in the oxygen consumption by the two subjects after 6 mg. per kilo was almost exactly twice the increase after 3 mg. per kilo. It is evident from inspection of the graphs that 1 mg. caffeine per kilo had no effect on the oxygen consumption as the total increase over the control experiments was only 150 cc. for one subject and 60 cc. for the other throughout the period of 1¾ hours.

The total heat production of W.W. for the entire experiment after taking 3 mg. and 6 mg. per kilogram body weight was, respectively, 4.9 and 16.7% higher than in the control experiments and that of C.E. 5.5 and 11.4% higher. These figures,

however, do not represent the total increase in heat production resulting from the ingestion of caffeine, for the rate of oxygen consumption at the conclusion of the experiment was still above that of the control experiments. Our results are of the same order as those of Means, Aub and DuBois ('17) who found an increase of 7 to 23% in the heat production of four normal subjects over a 2-hour period after taking 8.6 mg. caffeine per kilogram body weight. The smallest dosage of 1 mg. per kilogram in our experiments had no effect on heat production; the average difference in these and the control experiments was less than 1%.

Carbon dioxide elimination. The carbon dioxide in the expired air increased after taking caffeine in proportion to the amount ingested. One milligram per kilogram had no effect on the carbon dioxide elimination of C.E. and only a small effect on that of W.W. In the latter subject 270 cc. more carbon dioxide was eliminated after taking 1 mg. caffeine per kilogram than in the control experiments. Practically all the excess elimination occurred in the first and second post-ingestion periods. While it was relatively small it was sufficient to account for the rise in the respiratory quotient in the early part of the experiment as shown in the graphs. An interesting difference is observed in the carbon dioxide elimination and oxygen consumption curves with the largest dosage of caffeine. After reaching a peak, the former drop abruptly whereas the latter decline gradually.

Interpretation of the respiratory quotient. In order to interpret the rise in the respiratory quotient induced by caffeine, it is necessary to consider the various physiological processes capable of raising the quotient and to determine which might have been affected by caffeine. The various possibilities considered were: An increase in production and accumulation of lactic acid which would liberate carbon dioxide; stimulation of the respiratory center with a consequent hyperventilation; and an increase in carbohydrate oxidation in which case the quotient would be a true combustion quotient.

In these experiments there was no accumulation of blood lactic acid. In five of the experiments in which 6 mg. per kilogram was taken, blood was drawn from the antecubital vein at the conclusion of the third basal period and again at the end of the first post-ingestion period when the respiratory quotient was at or near the peak. The average basal blood lactic acid was found to be 11.3 mg. per cent and after taking

TABLE 1

Respiratory ventilation (liters per minute at 0°C. and 760 mm.) as affected by different amounts of caffeine[1]

SUBJECT W. W.

	BASAL	CONSECUTIVE 15-MINUTE PERIODS						
		1	2	3	4	5	6	7
Control expts. (200 cc. H_2O)	5.63	+ .32	+ .40	+ .24	+.03	—.15	+.13	+.32
1 mg. caffeine per kg.	5.45	+ .62	+ .66	+ .32	+.43	+.20	+.61	+.52
3 mg. caffeine per kg.	5.42	+ .82	+ .61	+ .44	+.56	+.54	+.81	+.60
6 mg. caffeine per kg.	5.69	+1.37	+1.41	+1.18	+.93	+.91	+.84	+.85

SUBJECT C. E.

	BASAL	CONSECUTIVE 15-MINUTE PERIODS						
		1	2	3	4	5	6	7
Control expts. (200 cc. H_2O)	5.81	+ .42	+ .29	+ .21	+.25	+.31	+.23	+.23
1 mg. caffeine per kg.	5.84	+ .48	+ .21	+ .09	—.01	+.08	+.02	+.03
3 mg. caffeine per kg.	5.46	+ .57	+ .92	+ .44	+.31	+.34	+.34	+.41
6 mg. caffeine per kg.	5.43	+1.35	+1.47	+1.13	+.59	+.51	+.78	+.86

[1] Each value in the table is an average obtained from five experiments.

caffeine 10.8 mg. per cent, the difference between the basal and post-ingestion samples in the individual experiments varying from 0.2 to 0.9 mg. per cent.

The generally accepted belief that caffeine acts on the respiratory center would suggest that the drug in sufficient amounts might induce hyperventilation with excessive loss of carbon dioxide. It is sometimes stated, however, that caffeine

increases the rate but not the depth of respiration (Cushny, '36). Under these circumstances it is conceivable that the stimulating action of caffeine on respiration might not induce hyperventilation. In interpreting the respiratory exchange in these experiments it is therefore necessary to take into account the minute rate and volume of respired air. These data are presented in tables 1 and 2. Since the results are more definite

TABLE 2

Average changes in the minute respiratory rate after taking caffeine [1]

SUBJECT W. W.

	BASAL	CONSECUTIVE 15-MINUTE PERIODS						
		1	2	3	4	5	6	7
Control expts. (200 cc. H_2O)	12.9	+1.3	+0.9	+1.2	+0.5	0.0	+0.2	+0.6
1 mg. caffeine per kg.	12.9	+0.3	+1.0	+0.6	+0.7	+0.5	+1.3	+0.5
3 mg. caffeine per kg.	12.6	+1.3	+1.1	+0.7	+0.9	+1.1	+1.6	+0.9
6 mg. caffeine per kg.	12.8	+1.3	+1.2	+0.8	+1.0	+1.8	+1.7	+1.6

SUBJECT C. E.

	BASAL							
Control expts. (200 cc. H_2O)	12.8	+0.2	+0.5	0.0	−0.8	−0.4	−0.4	−0.5
1 mg. caffeine per kg.	12.5	+0.3	−0.4	−0.2	−0.2	−0.2	−0.4	−0.6
3 mg. caffeine per kg.	12.3	+0.8	+0.9	+0.5	+0.9	+0.5	−0.2	+0.6
6 mg. caffeine per kg.	12.2	+0.4	+1.3	+1.3	+1.2	+0.9	+1.3	+1.6

[1] The minute respiratory rate was obtained by dividing by 15 the number of respirations recorded on a kymograph over 15-minute periods.

and unequivocal with the experiments in which 6 mg. of caffeine per kilogram was taken, these experiments will alone be considered in this interpretation. In these experiments the minute volume of expired air during the first three post-ingestion periods showed a much more pronounced increase than in the corresponding periods of the control experiments (table 1). The respiratory rate in these periods, however, was

not appreciably different from the rate in the controls (table 2). The depth of respirations and the tidal volume were therefore markedly increased. In the first and second post-ingestion periods the average increase in the tidal volume of the two subjects was 75 and 65 cc., respectively, whereas in the control experiments it was only 4 and 3 cc. In the last two periods of the experiments with the largest dose of caffeine the tidal volume had practically returned to normal; for the two subjects it was 10 cc. and 9 cc., respectively, above the basal as compared with 18 cc. and 15 cc. in the control experiments. Although the tidal volume was practically the same in the last two periods as in the control experiments, the minute volume of respired air was somewhat higher. This can be accounted for by the higher rate of respiration in the caffeine experiments during these periods. The larger minute volume as compared with the control experiments was most likely due to the increase in oxygen consumption which was still in evidence as shown in figure 1 at the conclusion of the experiment.

These observations on the respiratory exchange indicate that caffeine stimulated the respiratory center and thereby produced hyperventilation with excessive elimination of carbon dioxide. It may be concluded therefore that the rise in the respiratory quotient was due in part if not altogether to hyperventilation. Confirmatory evidence was obtained from determinations of the alveolar carbon dioxide. Three additional experiments were run on each subject in which alveolar air samples were obtained at the end of each 15-minute period by the method described by Carpenter and Lee ('33). In these experiments, which are not included in figure 1, the respiratory quotients followed the same general course as those in the graphs. The average basal alveolar carbon dioxide of W.W. was 5.93%. At the end of the first and second periods following the ingestion of 6 mg. caffeine per kilogram it had fallen to 5.40 and 5.51% and at the end of the fourth period had risen to 5.62%. Similar results were obtained with the other subject. The alveolar carbon dioxide percentage which was 6.42 in the basal periods fell to 6.12 and 5.99 in the first

and second periods after taking caffeine and rose to 6.23 in the fifth period.

The abrupt descent of the respiratory quotient in the third period which carried the quotient in the 6 mg. per kilogram experiments well below the base line, may be interpreted as indicating a compensatory retention of carbon dioxide after the hyperventilation which was responsible for the earlier rise in the quotient. It might be objected that this conclusion is not borne out by the course of the carbon dioxide elimination curves since they do not descend below those of the control experiments in the same manner as the respiratory quotient
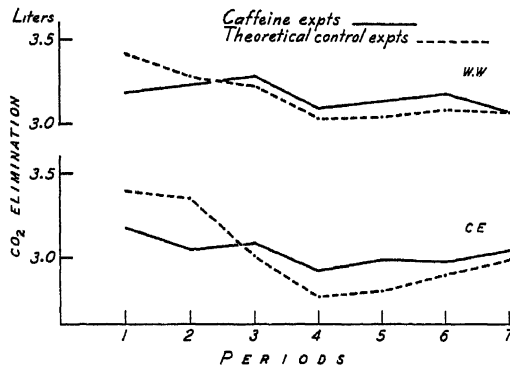
Fig. 2   Carbon dioxide elimination in the 6 mg. per kilogram experiments and in theoretical control experiments in which the same increase in oxidation as in the caffeine experiments has been assumed (see text).

curves. Further consideration, however, shows that this is not a valid objection. It is extremely important to take into account the fact that caffeine induced an increase in oxygen consumption. The curves of carbon dioxide elimination, there-fore, should not be compared with the curves actually obtained in the control experiments but with theoretical curves that would have been obtained if metabolism, as measured by oxy-gen consumption, had been stimulated to the same extent as in the caffeine experiments, the respiratory quotients remain-ing the same as those obtained in the control experiments. Such theoretical curves have been constructed and are pre-sented as solid lines in figure 2, together with curves plotted

from data in the 6 mg. per kilogram experiments. The latter curves are plotted as broken lines. It will be observed that the relationship between the curves is the same as that which obtained between the curves of the respiratory quotient in the 6 mg. caffeine per kilogram and control experiments (compare fig. 1). The carbon dioxide elimination confirms therefore the conclusion drawn from the respiratory quotient, namely, that caffeine produced a stimulation of the respiratory center which resulted in a blowing off of carbon dioxide. Later in the experiment there was a compensatory retention of carbon dioxide which was complete at the end of the experiment. An algebraic summation of the differences between the values represented by the two curves for the seven periods revealed a total difference of only 30 cc. for one subject and 40 cc. for the other.

There remains to take into account the possibility of an increase in carbohydrate oxidation concomitant with hyperventilation. When the total carbon dioxide elimination for the seven post-ingestion periods was divided by the total oxygen consumption in the various experiments, it was found that the post-ingestion respiratory quotient of W.W. in the control experiments was 0.01 above the basal and 0.01, 0.01 and 0.00 higher, respectively, in the 1, 3 and 6 mg. caffeine per kilogram experiments; the post-ingestion quotient of C. E. was 0.03 higher than the basal in the controls and 0.02, 0.03 and 0.02 higher, respectively, in the caffeine experiments. The rise in the respiratory quotients after taking caffeine consequently did not represent a stimulation of carbohydrate metabolism but was due entirely to hyperventilation. The proportion of the foodstuffs metabolized was not affected by the stimulating action of caffeine.

DISCUSSION

The results of these experiments differ from those reported by other workers in that caffeine in relatively large doses produced in our subjects a definite rise in the respiratory quotient. The difference in the results as compared with those of Means,

Aub and DuBois ('17) is probably due to the circumstance that our quotients were determined for 15-minute periods whereas theirs were calculated for periods of 1 hour. The rise of the quotients in our experiments, which is shown in figure 1, occurred in the first two periods and was followed in the next two periods by a descent below the basal level and also below the quotient in the corresponding periods of the control experiments. The rise was therefore largely counter-balanced by the subsequent drop. If the respiratory quotients had been calculated from the gaseous exchange of the first hour after taking caffeine, the increase above the basal for the two subjects in the experiments with 6 mg. per kilogram would have been only 0.02 and 0.04, respectively, as compared with a maximum of 0.06 and 0.10 in the first and second 15-minute periods.

In the other studies that report no rise in the respiratory quotient after taking caffeine, only one or two experiments were done on each subject and these experiments had no controls in which water alone was taken. It is our experience that it is hazardous to draw conclusions from so few experiments considering the unavoidable variations that are known to occur under the most carefully controlled experimental conditions. In confirmation of the work of others (Carpenter and Fox, '30) we have occasionally observed variations in the R.Q. of several points in either direction after taking 150 or 200 cc. of water at body temperature. These variations may be due to slight movements or to changes in muscle tone that cannot be readily detected (Carpenter, Hoskins and Hitchcock, '34).

Another factor that may influence the respiratory exchange is dozing on the part of the subject, for it has been shown that during sleep the oxygen consumption may decrease more than 15% (Mason and Benedict, '34). We have found that the respiratory quotient may also be affected. This may be accounted for by shallowness of respirations which may lead to hypoventilation. Training of the subject and watchfulness on

the part of the experimenter by using some simple device to make sure the subject stays awake are not always successful in preventing momentary sleep at some phase of a long experiment.

It follows from these considerations that complete reliance cannot be placed on the data of respiratory exchange unless averages are drawn from a sufficient number of carefully controlled experiments. If in spite of all precautions, an experiment is included in which vitiating factors were present but could not be definitely ascertained, the error thus introduced is greatly reduced and may become insignificant when an average is drawn from several experiments.

SUMMARY AND CONCLUSIONS

The effect of caffeine on the respiratory exchange has been studied on two subjects with dosages of 1, 3 and 6 mg. caffeine alkaloid per kilogram body weight.

One milligram caffeine per kilogram produced no effect on the oxygen consumption, carbon dioxide elimination or respiratory rate on either subject, nor in the respiratory quotient or total ventilation in one subject. In the other subject it produced a rise in the respiratory quotient and a slight increase in pulmonary ventilation.

The two larger doses were followed in the first and second 15-minute periods by a rise in the respiratory quotient, oxygen consumption and carbon dioxide elimination in both subjects. The rise in the quotient was followed by an abrupt descent which carried the quotient below the base line and below the level of the corresponding periods in the control experiments. The effects were more pronounced with the 6 mg. than with the 3 mg. per kilogram dose.

There was but little if any change in the respiratory rate following the ingestion of caffeine. In one subject there appeared to be a slight increase after the 6 mg. per kilogram dose. The tidal volume, however, showed a definite increase

with the 3 mg. and a pronounced increase with the 6 mg. per kilogram dose. The depth of respiration was therefore increased by the larger doses of caffeine, evidently the result of a stimulation of the respiratory center.

It is concluded that the rise in the respiratory quotient was due solely to the blowing off of preformed carbon dioxide.

Hyperventilation in the first two 15-minute post-ingestion periods was followed by a compensatory retention of carbon dioxide which was completed at the conclusion of the experiment.

The increase in metabolism produced by the larger doses of caffeine was not accompanied by a change in the proportion of the foodstuffs oxidized.

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SKIN TEMPERATURES OF THE PIG, GOAT, AND SHEEP UNDER WINTER CONDITIONS

ROBERT C. LEE, NICHOLAS F. COLOVOS AND ERNEST G. RITZMAN

*Nutrition Laboratory, Carnegie Institution of Washington, Boston, Massachusetts,
and New Hampshire Agricultural Experiment Station, Durham*

ONE FIGURE

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As the skin temperature is essentially a resultant of heat production and heat loss and as these two factors may be altered by wide ranges in environmental temperature, a study was made of the influence of relatively low (winter) environmental temperatures on the skin temperatures of animal species differing with respect to their protective coats and the insulating layers of fat under the skin. In the broad comparative studies of basal metabolism at the Laboratory for Animal Nutrition, of the University of New Hampshire, skin temperature measurements have already been made on steers (Benedict and Ritzman, '23, '27). The present report supplements the comparative picture with similar data for the goat, sheep, and pig, and these data have been compared with previous measurements of heat production of these same animal species under related conditions. As few skin temperature studies have been made on animals other than man and as our own data are not extensive, no attempt will be made to review the literature.

EXPERIMENTAL

A sow, a female goat, a ram, and a ewe, all adult, were used for the study. The ewe and the sow were pregnant. The time at which the animals last ate varied. The length of the hair of the sow and the goat at position 13 (see insert, fig. 1) was 5.1 cm. For the ewe and the ram the depth of the protective coat in its normal condition, at this point, was 8.9 and 5.4 cm., respectively.

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. The skin temperature was measured at the base of the hair or wool by a thermojunction, with the apparatus described by Benedict, Coropatchinsky and Finn ('28). The measurements were made on the right side at points selected to give a general picture of the average skin temperature. The results are shown graphically in figure 1. Points where few data were obtained because of the density of wool or the presence of moisture are omitted from the curves.

In this report the word "temperature" will be used to refer to skin temperature and the word "environment" to the condition of the surrounding air. All values are in degrees Centigrade. The cold environments were obtained in an animal shelter with one side open and the warmer environments in a room 3.6 by 4.6 meters, heated by a coal stove.

RESULTS

Constancy of results in two series of measurements. With the goat, under essentially similar conditions, two series of measurements (curves 3 and 4) were made an hour apart, at 18° and 21°. The temperatures on the same spot differed by 1.3° and 1.8° at two points on the head, but all others on any one spot agreed within 1°C. The average temperatures were the same. With the ewe two series at 14° (curves 7 and 8), made 20 minutes apart, showed a greater maximum variation at any one point, possibly due to local exposure in making the measurements, but the averages in these two series were only 0.3° apart. Usually measurements under like conditions could be duplicated within 1°C.

Adaptation to a given environment. Measurements were made on the goat immediately after it had walked about 18 meters from an environment of —12° to a room at 19° (curve 2) and again 1 and 2 hours after being in the warm room (curves 3 and 4). The first series agreed well with the two subsequent series, although averaging 0.3° higher (possibly because of the physical exertion of walking). Seventeen hours later, during which the environment had gradually fallen 5° lower, the temperatures (curve 5) averaged 2.3° lower. Hence the goat adjusted itself rapidly to changes in environment.

The pig, after observations in the barn at —12°, was measured again immediately after walking to the warm room at 19° and also 20 hours later (curves 11, 12, and 13). During these 20 hours the environment descended to 13°C. Although the later measurements were made at an environment 5° below the initial level in the warm room, the temperatures averaged 3.4° warmer than the initial temperatures. Hence with the pig the adjustment to changes in environment was not immediate, as it was with the goat.

Effect of differences in environment on the temperature of the same animal. Measurements on the ewe at 14° and — 3° (curves 7, 8, and 6) showed an average difference of about 5° in the temperature of the trunk, but even at the freezing environment (— 3°) the temperature on this portion of the body was still well above 30°C. The differences on the head were almost equivalent to the difference in environment. The average trunk temperature of the goat was 1.9° lower at 16° (curve 5) than at 21° (curve 4) but at —12° (curve 1) was only 4.5° below that at 16°C. The temperatures on the head at —12° were influenced to a greater degree by the environment than were those on the trunk. The temperature of the pig's trunk averaged 10° lower at — 3° (curve 10) than at 14° (curve 13), and the additional decrease of 9° in environment, i.e., to —12° (curve 11), resulted in a further decrease in trunk temperature of 4.5°C. The temperature of the pig's head was more uniform than that of the trunk, and at the —12° environment the head was generally warmer than the trunk.

Comparison of average temperatures of the different animals at various environments. Under essentially similar environmental conditions (14° to 16°) the temperatures of the sheep were higher on all portions of the body than those of the goat and the temperatures of the goat were higher than those of the pig. The average trunk temperatures at the different environments were as follows:

Environment	—12°	—3°	+3°	14°	16°	18°	21°
Goat	29.4°	—	—	—	33.9°	35.6°	35.8°
Sheep	—	31.7°	33.2°	36.3°	—	—	—
Pig	15.4°	19.9°	—	29.8°	—	—	—
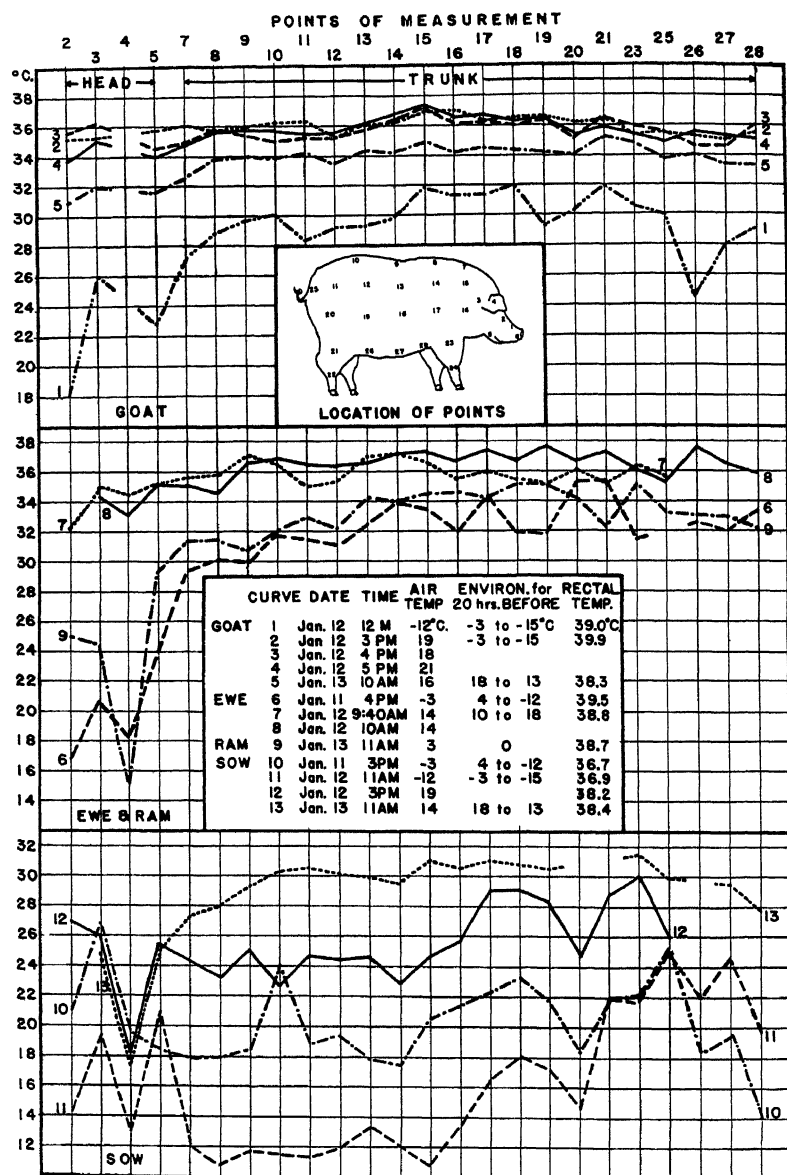
Fig. 1 Skin temperatures of farm animals on different parts of the body, as influenced by different environmental temperatures. The upper insert shows the positions of measurements for the sow; corresponding positions were used for the ram, ewe, and goat. The lower insert gives the conditions for the individual curves, air temperature indicating that during the measurement.

Clearly the sheep and the goat can maintain their temperatures relatively uniform over wide ranges in environment, as compared with the variability found with the pig.

Comparison of metabolism and skin temperature at different environments. Metabolism measurements made previously on similar adult animal species (Ritzman et al., '30, '31, '36) under contrasting environmental conditions, when quiet and fasting, show that the average heat production, expressed as calories per kilogram of body weight per 24 hours, is as follows:

SHEEP		GOAT [1]		PIG [1]	
°C.	Cals.	°C.	Cals.	°C.	Cals.
0 to 3	29.8	8	32.3	12	19.0
6 to 9	29.5	12 to 16	28.5	18 to 24	19.0
21 to 27	30.2	20 and over	23.9		

[1] Unpublished data obtained at the Laboratory for Animal Nutrition, University of New Hampshire.

The heat production of sheep was not affected by the environment, at least down to 0°. This, coupled with the finding that its temperature was essentially uniform over a wide range in environment, emphasizes the excellent protection afforded by the fleece. When the fleece was removed, the heat production increased. The metabolism of the goat increased immediately when the environment dropped below 20°, as was the case also with sheared sheep. Hence the relative uniformity in the goat's temperature even in a freezing environment may be ascribed both to its increased metabolism and to the protection afforded by its hair. The heat production of the pig remained the same at 12° as at 20° or over. Its skin temperature fluctuated with environmental changes, due to its poor hair coat, but it possessed a protection against cold in the form of large fat deposits in and under the skin. With this insulation its heat production remained uniform between 12° and 24°. These observations show that changes in skin temperature cannot be used as an indication of metabolic changes and that differences in the external protective coat and the fat insulation in and beneath the skin may account for wide variations in skin temperature not correlated with heat production.

SUMMARY

Skin temperature measurements were made on two sheep, a pig, and a goat at environmental temperatures between —12° and +21°C. Duplicate measurements on the same spot on the body usually agreed within 1°C. Equilibrium in skin temperature after change to a different environmental temperature was quickly established with the goat but more slowly with the sow. The ewe, the ram, and the goat maintained their skin temperatures near 30°, even when the environmental temperatures were at the freezing point or below. The skin temperature of the sheep was warmer than that of the goat or the pig under all conditions, the goat's temperature being slightly lower and the pig's appreciably lower. The average skin temperatures on the trunk were as follows:

ENVIRONMENT	GOAT	SHEEP	PIG
14° to 16°	33.9°	36.3°	29.8°
+ 3°	—	33.2°	—
— 3°	—	31.7°	19.9°
—12°	29.4°	—	15.4°

The protective coats of the sheep and the goat enabled them to maintain their skin temperatures within relatively narrow ranges despite large differences in environment, but the poorer external protection of the pig did not enable such uniformity.

Comparison is made between these skin temperature measurements and metabolism measurements previously made on similar animal species over a wide range in environmental temperatures.

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PEROSIS DUE TO A VITAMIN DEFICIENCY [1]

A. G. HOGAN, L. R. RICHARDSON, H. PATRICK AND
H. L. KEMPSTER

*Departments of Agricultural Chemistry and Poultry Husbandry,
University of Missouri, Columbia*

NINE FIGURES

Hogan, Guerrant and Kempster ('25) observed that chicks reared on simplified rations frequently developed the deformity of the legs later known as hock disease, slipped tendon, or perosis. Hogan and Shrewsbury ('30) observed that these symptoms are prevented by wheat or by wheat middlings and attempted to determine whether the protective action is due to mineral constituents. Accordingly wheat was ignited and the ash of 100 gm. of wheat was incorporated in 100 gm. of the experimental ration, but this addition was entirely ineffective. It was concluded that the abnormality was due to the deficiency of an organic constituent. In the meantime Wilgus, Norris and Heuser ('37 a) reported that perosis is due to a deficiency of manganese, an observation that has been amply confirmed.

At first it seemed difficult to reconcile these conflicting claims. Substances which in our experience prevented perosis lost their effectiveness when ignited. On the other hand the Cornell group almost entirely eliminated perosis merely by including a minute amount of manganese in the ration. In order to explain this discrepancy it was assumed that in addition to manganese an organic substance is required to prevent perosis. Our rations were deficient in the organic factor,

those of Wilgus, Norris, and Heuser were deficient in manganese. Hogan, Richardson, and Patrick ('40) were able eventually to obtain evidence that sustains our hypothesis. This paper is to present the evidence in more detail.

EXPERIMENTAL

Experimental procedure. The baby chicks were approximately 1 day old when delivered from the hatchery, and they were supplied with the experimental rations immediately. The experimental procedure has been described by Hogan and Boucher ('33).

Preparation of vitamin carriers. We had never succeeded in formulating a synthetic diet that is adequate for the chick unless liver extracts were included, and the chicks which received these rations seldom, or never, developed perosis. In order to detect the presence of an organic perosis-preventing substance it would be necessary to separate the liver extract into two fractions, one of which contains this hypothetical factor. The second fraction would not prevent perosis but it must contain the other vitamins that are necessary to maintain the chick in a reasonably satisfactory nutritional state.

The procedure as finally adopted is fairly simple:

1. Fresh beef liver is ground in a meat chopper, and dried in a current of air at a temperature of 55 to 60°C. The dried product is reground to a fine state of subdivision.

2. The dry liver powder is extracted at a temperature of 70°C. with 95% ethyl alcohol. The extracts are filtered hot and allowed to cool, when a considerable amount of lipoidal material separates and is discarded. The alcoholic solution is concentrated in vacuo until it begins to foam badly, then poured into cold water when a fatty fraction should rise to the top. Whether it does or not the mixture is warmed to 70°C. and then the fatty layer separates sharply and is skimmed off and discarded. The aqueous phase is filtered through several layers of cheese cloth and concentrated in vacuo to a dry matter content of about 50%. This is preparation 4303.

3. The liver powder residue from step 2 is extracted exhaustively with boiling 95% ethyl alcohol. This extract is not a highly active source of the perosis-preventing factor, and has not been extensively used.

TABLE 1

Composition of rations

CONSTITUENT	RATION NO.				
	4121	4507	4928	4970	4976
Casein	35	35	25	35	35
Corn starch	25	21	35	24	22
Cane molasses				5	5
Lard	7	7	15	15	15
Soybean oil	8	8			
Cellulose	3	3	3	3	3
Salts (3516)[1]	4	4	4	4	4
Cod liver oil	2	2	2		
A-D mixture [2]				2	2
Liver residue (4081)	10	10	10	10	10
Alcohol extract (4303)		4			
Water extract (4080)	6	6	6	2	4
MnSO$_4$, 4H$_2$O, gm.	0.1	0.1	0.1	0.1	0.1
Thiamine [3], µg.	200	200	200	200	
Riboflavin, µg.	400	400	400	400	
Vitamine B$_6$, µg.		300	500		
alpha-tocopherol, mg.			8	8	8
2-methyl-1,4-naphthoquinone, mg.			1	1	1

[1] Salts 3516, Hubbell, Mendel and Wakeman ('37).

[2] A concentrate of vitamins A and D dissolved in lard. According to the manufacturers' assay 1 gm. of the mixture contains 3000 units of vitamin A and 425 units of vitamin D.

[3] The vitamins were generously supplied by Merck and Co., Rahway, New Jersey.

4. The liver powder residue from step 3 is extracted with tap water previously heated to boiling. The extracts are combined, filtered, and concentrated in vacuo to a dry matter content of about 50%. This is preparation 4080.

This preparation should contain little or none of the perosis-preventing factor, but in order to attain a satisfactory degree of separation it is necessary that the preceding extraction with alcohol be complete.

5. The residue remaining after extraction with hot water is preparation 4081.

Typical experimental rations are described in table 1.

The alcohol extract of liver prevents perosis. Since the extent of the deformity is variable the degree was estimated by the method of Wilgus, Norris and Heuser ('37 b). According to this system the maximum degree is 100.

The response of the chicks to the two types of liver extracts is shown in table 2. Ration 4121 contains the water extract of

TABLE 2

Response of chicks to various rations, at close of a 6-week experimental period

GROUP AND RATION	BREED	FEMALES		MALES		PEROSIS		MOR-TALITY
		No.	Weight	No.	Weight	De-gree	Inci-dence	
			gm.		*gm.*	%	%	%
I 4121	W.L.[1]	15	240	13	198	67	100	37
	N.H.R.	4	268	1	258	51	100	0
	W.R.	12	208	11	164	57	100	30
II 4121 + 300 μg. vitamin B_6	W.L.	1	290	2	412	46	100 ·	
	N.H.R.	2	572	1	654	52	100	
	W.R.	6	333	3	406	66	100	
III 4121 + 500 μg. vitamin B_6	W.L.	3	384	8	405	33	100	
	W.R.	5	470	6	480	52	100	
IV 4507	W.L.	7	410	7	459	0	0	
	N.H.R.	2	553	3	549	0	0	
	W.R.	5	512	6	640	0	0	
V 4121, Mn omitted	W.R.	3	183	3	218	48	100	
	N.H.R.	2	108	2	215	97	100	25
VI 4121, Mn omitted + 500 μg. vitamin B_6	W.R.	3	250	3	358	92	100	
VII 4507, Mn omitted	W.R.	2	549	2	583	0	0	
VIII 4121, Mn at minimum	W.R.	6	150	6	126	47	100	41
	N.H.R.	2	287	2	289	81	100	0
IX 4121, Mn at minimum + 500 μg. vitamin B_6	W.L.	4	295	6	319	80	100	
	W.R.	3	285	6	320	80	100	
X 4507, Mn at minimum	W.L.	5	397	5	450	14	70	
	N.H.R.	2	540	2	625	48	100	
	W.R.	6	461	6	609	8	8	

[1] W.L.=White Leghorn.   N.H.R.=New Hampshire Reds.   W.R.=White Rocks.

liver but not the alcohol extract. Symptoms of perosis appeared at the end of the second week and by the end of the fourth week all chicks which survived had well developed symptoms. The chicks grew slowly and the mortality was high. Since the symptoms suggested that the mortalities were due to a deficiency of vitamin B_6, this vitamin was added to the basal ration (groups II and III). Addition of 300 or 500 µg. largely eliminated the mortalities, and accelerated the growth rate, but it did not reduce either the incidence or degree of perosis. Apparently the chick requires between 300 and 500 µg. of vitamin B_6 for the optimum rate of growth.

The chicks just described received the water extract of liver and developed perosis. The next group received ration 4507 which contains both the water and alcohol extracts, and every chick was normal. An example of perosis is shown in figure 9.

Effect of a manganese deficiency. According to Gallup and Norris ('39) the amount of manganese required for normal bone development is approximately fifty parts per million. A study was made, therefore, to determine the effectiveness of the alcoholic extract when the ration is deficient in manganese. The first ration with a low manganese content (group V) contains twenty parts per million. This ration contains the water extract of liver but not the alcohol extract. Symptoms of perosis appeared before the end of the second week and by the end of the third week the condition was well developed. The chicks grew slowly and the mortality was high. Again there was reason to believe that the mortalities were due to a deficiency of vitamin B_6, and therefore 500 µg. per 100 gm. were added to the diet (group VI). The addition largely eliminated the mortalities and accelerated the growth rate, but it did not reduce either the incidence or degree of perosis.

Group VII received a ration which contains both the water and alcohol extracts. It will be observed that the incidence of perosis is zero, and of course the degree is zero. The only breed of chicks tried was the White Rock. In this case perosis was completely prevented by supplying the chicks with the

alcoholic extract of liver though the manganese level was approximately 30 p.p.m.

An attempt was now made, with little success, to prepare a ration (group VIII) with a minimum manganese content by omitting manganese from the salt mixture. The new mixture was the same as mixture 3516 in all other respects. However, as shown in table 3, the amount of manganese in mixture 3516 was so low that omitting it entirely had no detectable effect on the analysis. The ration of group VIII contained the water extract of liver but not the alcohol extract. The chicks grew slowly, therefore vitamin B_6 was added at a level of 500 µg. per 100 gm. (group IX). This addition brought about vast improvement in the condition of the chicks but the incidence and degree of perosis was about the same as when the vitamin was omitted.

Group X received both the water and alcohol extract but again the manganese content was reduced to a minimum by omitting all inorganic compounds of this element from the ration. It will be observed that the incidence of perosis was 70% for the White Leghorns, 100% for the New Hampshire Reds and only 8% in the White Rocks. Apparently breeds that mature early are more subject to perosis. It also seems evident that when the ration is deficient in manganese, perosis cannot be prevented by supplying the alcoholic extract, though this constituent does reduce the degree of severity. Both manganese and the extract are required to entirely eliminate this abnormality.

The data have been scrutinized to see whether susceptibility to perosis is affected by sex, but this factor is apparently of little or no significance. It is our experience that when the ration is deficient in the organic perosis-preventing substance all breeds are equally susceptible. As was previously mentioned though, there is a differential breed susceptibility when manganese is deficient.

Substitution of other minerals for manganese. According to Wilgus, Norris and Heuser ('37 a) the addition of iron, zinc, and aluminum to perosis-producing rations is somewhat

effective in reducing the severity of the symptoms. These elements, each at a level of 0.0025% of the ration, were added to ration 4121. These additions increased the amounts of iron and zinc in the complete ration only slightly, but increased the total amount of aluminum by almost 70%. This modification did not affect either the incidence or the degree of perosis. Lyons, Insko and Martin ('38) also obtained negative results with these elements.

In the past perosis has been ascribed to the provision of an insufficient quantity of manganese, or to the provision of excessive amounts of calcium and phosphorus, or to unsuitable proportions of the mineral elements. For that reason we are reporting in table 3 a partial mineral analysis of the rations used to demonstrate that perosis was due to deficiency of an organic factor. It will be observed that the rations which prevent, or do not prevent, perosis may contain essentially the same percentages of the inorganic constituents. It is especially significant that a ration such as no. 4121 may contain five times as much manganese as the chick requires, and still permit every one that consumes it to develop perosis.

TABLE 3

Mineral content of rations [1]

GROUP AND RATION	CALCIUM	PHOS-PHORUS	MAN-GANESE	IRON [2]	ZINC [2]	ALUMINUM [2]
	%	%	%	%	%	%
I 4121	0.86	0.99	0.027	0.037	0.028	0.0036
IV 4507	0.86	1.02	0.028	0.048	0.032	0.0017
V 4121, Mn omitted	0.86	0.99	0.002	0.037	0.028	0.0036
VII 4507, Mn omitted	0.86	1.02	0.003	0.048	0.032	0.0017
VIII 4121, Mn at minimum	0.86	0.99	0.002	0.037	0.028	0.0036
X 4507, Mn at minimum	0.86	1.02	0.003	0.048	0.032	0.0017

[1] Determined by calculation from the analysis of the individual constituents.
[2] Determined spectrographically by Dr. V. R. Ells.

Measurements of bones. Caskey, Gallup and Norris ('39) have shown that chicks with perosis have leg and wing bones that are abnormally short and thick. Since the dimensions provide an objective criterion of perosis a considerable number of measurements were taken. The chicks were White Rocks with the exception of six which were New Hampshire Reds. The chicks were sacrificed and the humerus, tibia, and metatarsus were immediately dissected out with the cartilage caps in place. At first the bones of both the left and right sides were measured, but since the measurements of corresponding bones of the same chick were practically identical the later practice was to take measurements on only one side. The length of the humerus was obtained by measuring the distance between the trochlea and the oval articular head at the proximal end. The length of the tibia was obtained by measuring the distance between the distal and proximal extremity and the length of the metatarsus was obtained by measuring the distance between the trochlea for inner digit and the proximal extremity. The diameter was measured in the region of the shaft that appeared to be the most narrow. The shaft was placed in the calipers and turned, the highest reading being recorded.

Most of the chicks with perosis were smaller than the controls and after eliminating those in which the disparity in size was too pronounced only eleven pairs remained that had approximately the same weight. The weights of the twenty-two chicks ranged from 180 to 654 gm. The average weights and measurements of the eleven pairs on the two diets are shown in table 4. The differences between the two sets of

TABLE 4

Weights and bone measurements of chicks on the control and on the perosis-producing ration

RATION NO.	WEIGHT PER CHICK	LENGTH			DIAMETER		
		Humerus	Tibia	Metatarsus	Humerus	Tibia	Metatarsus
	gm	*mm.*	*mm*	*mm.*	*mm.*	*mm.*	*mm.*
4121	382.7	48.6	69.2	50.1	5.6	6.0	6.8
4507	383.6	54.8	75.3	54.5	5.1	5.0	5.9

measurements are entirely consistent. In every case the bones of chicks which did not receive the alcohol extract were shorter and thicker than those from chicks which did receive it.

The measurements of the tibia from chicks on four different rations are plotted against weight in figures 1 to 8. In some cases the variability is so great that the exact position of the smoothed curves is of no great significance, but they have been drawn to facilitate comparisons. The chicks that received
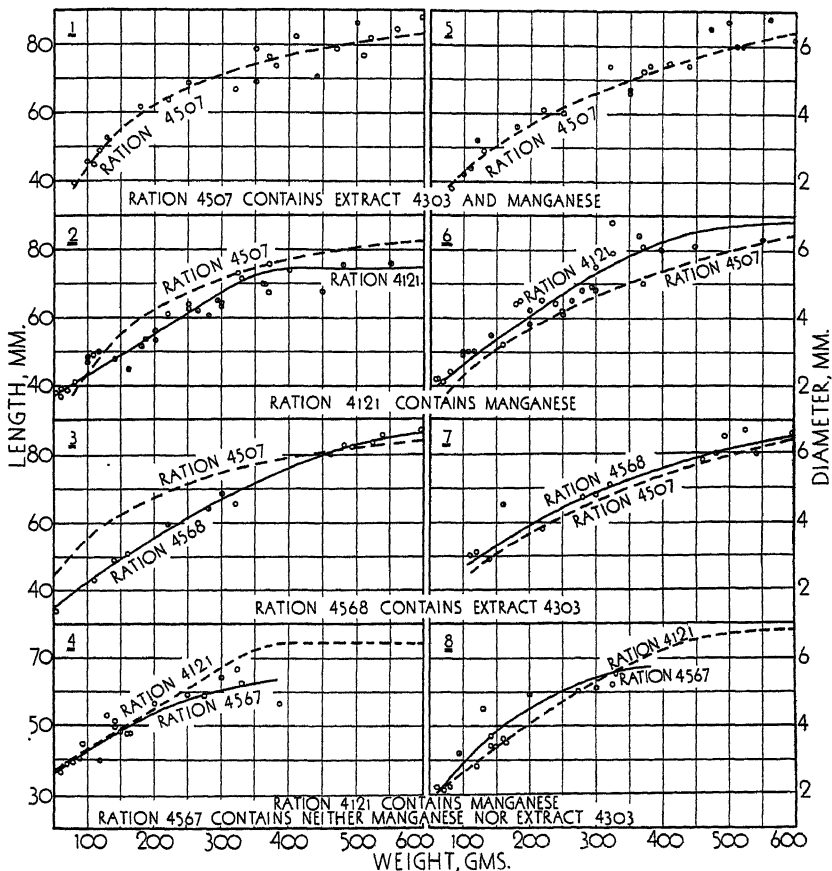
Figs. 1 to 8 Measurements of tibia, from White Rocks. The data points for chicks on ration 4507 appear only in figures 1 and 5, for chicks on ration 4121 they appear only in figures 2 and 6.

It will be noted that when the alcohol extract is omitted the abnormality is most marked. When only manganese is deficient the degree of perosis is intermediate. Ration 4567 was supplied to group VIII, ration 4568 to group X, of table 2.

ration 4507, which contained the alcohol extract and liberal amounts of manganese, were normal, and their bones were taken as a standard.

Ration 4121 contained a liberal amount of manganese, but did not contain the alcohol extract. The bones of chicks which received this ration were shorter and thicker than normal.

Ration 4568 contained the alcohol extract but was deficient in manganese. At the lower weights (and ages) the bones were abnormally short, and to a lesser degree they were abnormally thick. At the higher weights (6 weeks of age) the bones were normal in length and apparently they were also normal in diameter.

Under the experimental conditions described it seems that a deficiency of the alcohol extract alone, was more decisive in producing abnormal bones than was a deficiency of manganese, but it may be that the deficiency in the mineral was not sufficiently acute. Gallup and Norris ('39) report that perosis in White Leghorns was completely prevented on rations that contained thirty parts per million of manganese.

Ration 4567 did not contain the alcohol extract and was deficient in manganese. As would be expected the bones were abnormally short and abnormally thick. A comparison of these chicks with those which received ration 4121 would be of interest since neither ration contained the alcohol extract, but the comparison is difficult because the deficiency of manganese depressed the rate of growth. Such data as are available indicate that the bones of chicks which received ration 4567 were distinctly the shorter at the higher weights; the diameter was apparently greater at the lower weights, and less at higher weights. The differences are too uncertain to be of much significance.

On gross inspection the symptoms of perosis are highly variable. In the same group of chicks it may be observed that: (1) The tendon of Achilles is out of position, and the tarso-metatarsal bone is rotated in such a way as to point the toes to one side; (2) the tibial-tarsometatarsal joint is greatly enlarged; (3) the tarsometatarsal bone is markedly short and

thick; (4) because of curvature of the tarsometatarsal bone the chicks are bow-legged. Curvature of the tibia is less pronounced; (5) the long bones are all shorter and thicker than is normal, but the deformity would pass unnoticed on casual examination. In our experience the fifth set of symptoms is by far the most common. Each of the first four may be exhibited by a small per cent of the total number, though an occasional chick will develop them all. This wide variety of symptoms suggests the possibility that the organic factor concerned is multiple in nature.
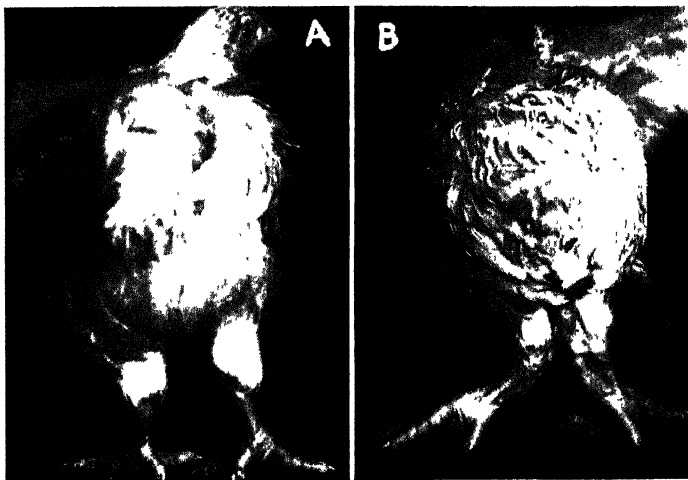
Fig. 9 A, normal chick. B, example of perosis. The joints and bones are enlarged, and the tendon of Achilles on the left leg is out of position.

Choline as a perosis-preventing agent. It has been shown by Griffith and Wade ('39) and by Abbott and De Masters ('40) that choline has unique physiological properties and it was included in some of the perosis-preventing rations. In the meantime Jukes ('40) had shown that choline at a level of 0.15 to 0.30% prevents perosis in turkeys.

Several groups of White Leghorn chicks received perosis-producing rations, and others received the same ration but with 0.1% of choline added. At the close of the experimental

TABLE 5

The effect of choline on bone development

GROUP AND RATION	CHICK NO.	WEIGHT	LENGTH			DIAMETER		
			Humerus	Tibia	Meta-tarsus	Humerus	Tibia	Meta-tarsus
			mm.	mm.	mm.	mm.	mm.	mm.
4976	414M	460	52.0	77.7	55.1	5.4	5.5	5.8
A 4976 +								
choline	418M	470	53.4	83.2	59.2	4.9	4.6	5.4
Normal[1]	347M	485	56.5	80.5	58.2	5.0	4.9	5.7
4976	414M	460	52.0	77.7	55.1	5.4	5.5	5.8
B 4976 +								
choline	420M	495	55.3	81.0	59.3	5.6	5.8	6.4
Normal	347M	485	56.5	80.5	58.2	5.0	4.9	5.7
4976	416F	362	47.7	68.0	48.9	5.0	5.6	5.8
C 4976 +								
choline	421F	370	51.6	75.0	53.5	4.8	4.1	5.2
Normal	346F	368	49.9	68.5	50.2	4.7	4.3	5.4
4976	417F	335	43.8	65.2	45.5	6.1	5.4	6.2
D 4976 +								
choline	422F	300	49.6	70.3	50.1	4.6	4.2	4.9
Normal	436F	320	49.4	72.6	50.1	4.1	3.9	4.6
4976	414M	460	52.0	77.7	55.1	5.4	5.5	5.8
E 4970 +								
choline	363M	465	54.4	78.6	58.1	4.9	5.5	5.9
Normal	347M	485	56.5	80.5	58.2	5.0	4.9	5.7
4976	414M	460	52.0	77.7	55.1	5.4	5.5	5.8
F 4970 +								
choline	366M	500	52.8	76.1	56.6	5.2	5.5	6.1
Normal	374M	505	58.7	88.7	65.5	5.1	5.0	5.5
4970	361F	252	38.0	53.7	38.6	4.8	4.4	5.0
G 4970 +								
choline	364F	290	42.7	62.8	42.9	4.6	4.5	4.9
Normal	437F	245	47.5	66.2	47.6	4.4	4.0	4.7
4970	358F	340	46.8	68.8	50.1	4.8	4.6	4.8
H 4976 +								
choline	421F	370	51.6	75.0	53.5	4.8	4.1	5.2
Normal	346F	368	49.9	68.5	50.2	4.7	4.3	5.4

[1] The ration contains the alcohol extract, and bone development should be normal. These rations are not described in the text.

period the chicks were sacrificed and three of the long bones were dissected out and measured.

The ideal comparison would be between three types of diets, (1) perosis-producing, (2) the same as (1) but with choline added, and (3) a diet that contains the alcohol extract and is known to prevent perosis. Unfortunately chicks of suitable weight for comparison were not available in every case. The ideal was attained in five of the trials appearing in table 5. In three of them, E, F, and H, the first two diets differed not only in content of choline, but also in the content of the water extract, no. 4080. It is uncertain whether this latter difference is the explanation, but it will be noted that in one trial, F, choline failed to bring about normal bone development. In trial G the second ration is the same as the first, excepting the presence of choline, but bone development of the chick which received choline is intermediate between the one which did not, and the one which received the alcohol extract. With the exceptions noted the data are fairly consistent and there is no doubt that choline has perosis-preventing activity. However, it remains to be seen whether it is identical with the active agent present in liver, or in other effective natural foodstuffs.

The weights of the chicks were not markedly increased by supplying choline, though the data were too few and variable for any precise distinction. When the rations are entirely adequate in all other respects the differences might be more conspicuous.

SUMMARY

1. A procedure was devised by which perosis can be produced uniformly in the chick, even when manganese is supplied in abundance.

2. This type of perosis is due to the absence of a specific organic nutrient. Choline is such a nutrient, but it was not shown that it is the only one.

3. Under the procedure followed the supply of calcium, phosphorus, manganese, iron, aluminum, or zinc was not responsible for either the incidence or degree of perosis.

4. The long bones of perotic chicks are abnormally short and thick.

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ARSENIC AND GOITER

GEORGE R. SHARPLESS AND MARGARET METZGER

Department of Laboratories, Henry Ford Hospital, Detroit, Michigan

In a recent paper Scott ('38) has collected data which provide strong circumstantial evidence of a goiterogenic role for arsenic. Arsenic through its action of combining with sulphydryl groups and therefore impairing one of the oxidation reduction systems of the body might influence thyroid activity. There are numerous reports of an antagonism between arsenic and thyroid. Hesse ('33) in one of the most recent studies found that arsenic limited the toxicity and prevented death in animals poisoned by thyroxin and thyroid. If arsenic is goiterogenic there should be a high incidence of goiter in Styria, the home of the arsenic eaters. Scott ('38) states that "the incidence of goiter, cretinism and deaf-mutism in that region (Styrian Alps) is tremendous". Furthermore there is a high incidence of goiter in Cordoba province, Argentina, where there are several endemic loci of poisoning from arsenical waters.

Arsenic is used extensively as an insecticide and although most of the spray may be removed from the surface of the food before it is eaten, much of the material eventually reaches the soil of the field in which it is used where it is a potential contaminant for any vegetation that might grow there. In the treatment of syphilis arsenic compounds play a major role and arsenic with iron is probably still used extensively as a tonic. Since the body is likely to obtain considerable arsenic from one source or another, its relation to goiter should be determined.

In a previous study (Sharpless, Pearsons and Prato, '39), rats fed a soy bean flour diet with 4.0 µg. of iodine added per 100 gm., had thyroids twice the normal size. Such rats should be very sensitive to any goiterogenic substance. This is a report of the effect of arsenic on the thyroid size and iodine content of rats fed this diet.

EXPERIMENTAL

The experimental procedures were the same as those previously described (Sharpless, Pearsons and Prato, '39). Young rats, both male and female, weighing 60 to 80 gm., were used in each experiment. Although the thyroid weight per unit of body weight is the same for both sexes, males and females were distributed evenly between the diets. The percentage composition of the basal diet was as follows: Unprocessed soy bean flour 75; sucrose 15.5; butterfat 5; dried yeast 3;

TABLE 1

Supplements to the diets

	DIET NO.					
	208	210	278	279	282	285
KI, µg. "I"/100 gm.	4.0	50.0	4.0	4.0	50.0	4.0
Arsenic pentoxide, mg./100 gm. "AS"	—	—	5.0	20.0	20.0	—
Arsenic trioxide, mg./100 gm. "AS"	—	—	—	—	—	20.0

sodium chloride 1; calcium carbonate 0.5. Supplements of iodine supplied as KI, arsenic acid and arsenous acid were added to this mixture as shown in table 1.

The arsenic acid was added in water solution while the arsenous acid, being only slightly soluble, was added as a dry powder. The control animals were given the basal diet with addition of 4 µg. of iodine per 100 gm. (diet 208) or 50 µg. of iodine per 100 gm. (diet 210). The experimental animals which were litter mates of the controls, were given the same diets with the addition of arsenic.

RESULTS

The average gain in body weight which can be used as an indication of toxicity, the thyroid weight and iodine content

are shown in table 2. Since similar results were obtained in all of the control groups fed diet 208 for 7 weeks, they were combined and tabulated as one group.

Fifteen rats fed diet 278 with 0.3 to 0.4 mg. arsenic intake per day had thyroids that weighed 19 mg. per 100 gm. of body weight compared with 17 mg. for controls fed diet 208. The results were analyzed mathematically according to the method described by Dunn ('29). The analysis indicated that there were eight chances in a hundred of obtaining this difference by pure chance and therefore the difference is not significant. Though the iodine content of the glands was slightly decreased,

TABLE 2

Thyroid weight and iodine content

DIET	NO. OF RATS	LENGTH OF EXPER.	AVERAGE GAIN IN WEIGHT	AVERAGE WET THYROID WEIGHT		SIGNIFI-CANCE RATIO TO CON-TROLS	PROBA-BILITY	IODINE IN THYROID	
				Total	Per 100 gm. body weight			Average total	Dry weight
		days	*gm.*	*mg.*	*mg.*		%	*μg.*	%
208	54	49	95	26	17 ± 0.4			2.1	0.033
278	15	49	95	31	19 ± 0.7	1.72	8.0	1.8	0.024
279	31	49	54	28	22 ± 0.6	4.7	0	1.1	0.018
285	14	49	81	27	17 ± 0.6	—	—	2.3	0.040
208	6	81	119	32	18 ± 1.7	—		—	—
278	6	81	112	36	19 ± 0.6	—		—	—
210	12	49	98	13	8 ± 0.4			6.6	0.166
282	15	49	76	14	10 ± 0.4	2.21	2.8	5.6	0.107

body growth was unaffected. Similar results were obtained with six rats kept on the diet for 81 days instead of the usual 7 weeks. With four times as much arsenic (diet 279) thyroids which weighed 22 mg. per 100 gm. of body weight were obtained. There is less than one chance in a million that this is fortuitous and therefore this is a significant difference. Body growth and thyroid iodine concentration were approximately 50% of the values obtained in the controls.

While the total thyroid weight was not increased, a comparison of rats of different weights can be made only on the thyroid weight per unit of body weight. A greater demand on the thyroid and consequently a larger gland would be ex-

pected in animals with the greatest growth providing the iodine content of the diet remains at the same level. According to published data, Remington's ('37) as well as our own, this is true. Therefore, if diet 279 were not goiterogenic we would have expected the thyroids to be smaller on the basis of total weight, as well as per unit of body weight, than those of rats fed diet 208. Since total thyroid weight was slightly greater and the weight per unit of body weight was significantly increased, while growth was limited, the conclusion is that arsenic can act as a goiterogenic substance.

Except that it was in trivalent form, the same concentration of arsenic as in diet 279 was fed to fourteen rats (diet 285). Thyroid weight and iodine content were almost identical with the values obtained for controls. The decreased rate of growth is not significant because the growth of litter mate controls of this group was also low. The results were unexpected and can be explained with the present data only on the basis of the insolubility of arsenous acid. Since the toxicity of arsenous acid depends upon the size of the particles (Schwartze, '22), it is probable that insufficient arsenic was absorbed to exert either a toxic or goiterogenic effect.

In order to determine whether the goiterogenic action could be prevented by iodine and also whether iodine would influence the toxicity of arsenic, 20 mg. of arsenic and 50 µg. of iodine per 100 gm. of diet were fed in diet 282.

The same amount of iodine was fed in the control diet 210. Thyroids weighing 10 mg. per 100 gm. of body weight were obtained in the arsenic-fed rats compared with 8 mg. per 100 gm. of body weight for their controls. Mathematical analysis indicated that there were approximately three chances in a hundred of this difference being fortuitous. In view of the variation that occurs in the thyroid weight, it is questionable whether this is a significant difference in weight. However, the thyroid iodine content was reduced to nearly 0.1% of the dry weight, the minimum content compatible with normal structure. Growth was approximately 80% of that of controls which was better than the 50% obtained in diet 278. Thus, arsenic was less toxic in the presence of higher iodine intake;

it caused increase in the size of the thyroid, of probable but not proven significance; it definitely reduced the iodine content and concentration of the thyroid.

DISCUSSION

These experiments show that arsenic can act as a positive goiterogenic substance and that iodine can reduce the goiterogenic action as well as the toxicity of small amounts of arsenic. The iodine requirement is increased appreciably. The total iodine content of diet 282 was approximately 55 µg. per 100 gm., nearly six times the 9.5 µg. per 100 gm. contained in our normal stock diet that gives an equivalent thyroid iodine content. Previous work has shown (Sharpless, Pearsons and Prato, '39) that rats fed soy bean flour require 20 to 25 µg. of iodine per 100 gm. of diet to produce thyroids containing 0.1% iodine. When this increased requirement is taken into consideration, the iodine requirement of rats fed diet 282 is more than twice that of rats fed the same diet without arsenic.

Can arsenic act as a positive goiterogenic agent in man? If the results obtained with rats give a direct indication, the answer is a qualified "no". They show that under ideal conditions for exerting a goiterogenic action, arsenic in nontoxic amounts has an insignificant effect. However, the results also indicate that in those areas where the iodine intake is relatively low, a goiterogenic effect can be expected if the arsenic intake is sufficient to be slightly toxic. While it is well known that people can consume huge doses of arsenic when it is eaten in large particles there is little experimental evidence to support the belief that a great tolerance for it can be acquired. Schwartze ('22) reviewed animal experiments as well as reports on the arsenic eaters and concluded that the so-called habituation to arsenic can be explained more readily on the basis of non-absorption from the intestinal tract than on the basis of an increased physiological tolerance for the poison.

The mechanism by which arsenic exerts its goiterogenic effect may be indirect, that is, by interfering with some of the normal oxidation-reduction systems. Glutathione decreases the toxicity of arsenic (Rosenthal and Voegtlin, '30). This

suggests that glutathione and possibly other sulphydryl containing compounds of the living cells are involved in a chemical union with arsenic. Barry, Bunbury and Kennaway ('28) have shown that arsenic has a retarding action on at least three oxidation-reduction systems. If by combination with the sulphydryl group or some other means, arsenic can hinder cell oxidation, then the thyroid might be stimulated by an effort of the body to compensate for lost oxidative capacity.

SUMMARY

1. Arsenic fed in non-toxic amounts (0.005% of the diet) had a slight, but not significant goiterogenic effect.

2. When the concentration of arsenic was 0.02% of the diet, growth was decreased 50%, iodine concentration of the thyroid was decreased and the thyroid weight was significantly increased.

3. Five times the minimum requirement of iodine when fed with 0.02% arsenic produced an enlargement of questionable significance, but lowered the thyroid iodine concentration.

4. It was calculated that 0.02% arsenic in the diet more than doubled the iodine requirement.

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CHEMICAL ESTIMATION OF QUALITY IN ANIMAL PROTEIN CONCENTRATES

H. J. ALMQUIST

Division of Poultry Husbandry, University of California, College of Agriculture, Berkeley

ONE FIGURE

In an earlier report (Almquist, Stokstad and Halbrook, '35) details were given of a chemical method for the estimation of quality in animal protein concentrates. Good agreement was shown between chemical and biological data obtained on a variety of samples. In the present report it is desired to add some further results of a supporting nature.

PROCEDURE

Chemical investigations of protein concentrates were conducted as already described (loc. cit.). The biological testing procedure was also very similar to that formerly used. White Leghorn chicks were used in all tests.

The basal diet contained wheat bran 10 parts, ground wheat 20, ground yellow corn 20, corn starch 25, dried alfalfa 4, dried brewer's yeast 5, oil [1] 3, sardine oil $\frac{1}{2}$, salt, plus small amounts of manganese, iron and copper 1, limestone $1\frac{1}{2}$. To 90 parts of this basal ration were added the animal protein supplement in amount sufficient to provide 6 parts of crude protein, and bone meal to adjust the total bone meal content of the ration to 3 parts. The total protein level of the completed ration was close to 15% in all cases.

As a biological measure of the quality of the protein concentrate, the gain per unit of food consumed from 4 to 6

[1] Wesson.

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weeks of age was selected as being more free from the influence of seasonal and other factors which may affect total gain. Chemical and biological results obtained are given in table 1. This table includes the data of three separate experiments in each of which the same standard sample of sardine meal was included. The gain per unit of feed consumed in the case of the sardine meal was 0.300 in one test and close to this figure

TABLE 1

Chemical and biological data on animal protein concentrates

SAMPLE NO.	DESCRIPTION	PERCENTAGE OF SAMPLE AS PROTEIN					PROTEIN QUALITY INDEX	GAIN PER UNIT FEED
		Total crude	Copper-precipitable	Undigestible	Phospho-tungstic acid-precipitable	Hot-water-soluble		
86	Sardine meal	69.3	67.0	5.0	0.6	3.1	87.2	0.300
87	Shark meal	74.0	65.5	20.1	3.1	6.2	58.1	0.197
88	Shark meal	81.1	68.3	4.9	1.5	5.4	75.0	0.263
89	Sardine ''stick,'' dried	68.3	35.1	1.6	8.7	28.4	29.3	....
90	Blend, 8 parts 86, 1 part 89	....	....	....	....	....	80.7[1]	0.281
91	Menhaden ''stick,'' dried	62.3	37.6	3.3	6.5	25.2	34.8	....
92	Blend, 8 parts 86, 1.1 parts 91	....	....	....	....	....	81.2[1]	0.284
93	Cartilage, dried	74.8	68.9	5.9	0.0	16.0	71.4	0.248
94	Blend, 4.4 parts 86, 4.0 parts 93	....	....	....	....	....	79.3[1]	0.260
95	Gelatin	91.9	91.9	0.0	0.0	91.9	40.0	0.150
96	Blend, 4.5 parts 86, 3.3 parts 95	....	....	....	....	....	63.6[1]	0.237

[1] Calculated from the indices of the separate concentrates.

in the other two. In these latter two tests the gain coefficients were slightly corrected throughout by simple proportion to a value of 0.300 for the standard sardine meal.

The protein quality index was calculated for each sample as in the former report. This calculation may bear repeated explanation. It consists of subtracting from the copper-precipitable protein (table 1) all of the undigestible protein and

0.6 of the hot-water-soluble protein. To the result is added 0.4 of the phosphotungstic acid-precipitable protein, and the final sum is divided by the total crude protein of the sample and multiplied by 100. Reasons for these steps and for the coefficients were given in the earlier paper. The relation of the protein quality index of the supplements or blends to the gain per unit of feed consumed is shown in figure 1.

As discussed in detail in the former report, the chemical analysis affords an insight as to the nature of the differences in quality that exist between samples. On the other hand, a
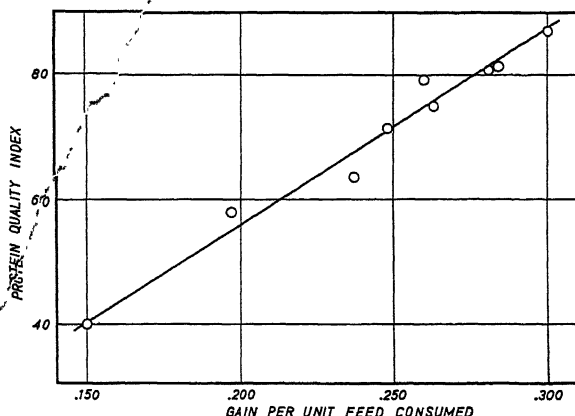
Fig. 1   Relation of protein quality index to gain per unit of feed consumed.

biological test merely demonstrates a difference without disclosing the underlying causes of the difference. For example, it is evident from the analyses that the principal point of inferiority of sample 87, as compared to sample 88, both shark meals, is a high content of undigestible protein. This is reflected in the index and also in the gain coefficient.

Dried fish meal "stick" shows a very unfavorable analysis, · featured particularly by a high content of hot-water-soluble protein quite similarly for samples 89 and 91. Blends of these samples with the standard sample 86 gave lower gain coefficients in direct relation to lower protein quality indices of the blends. These results indicate that the blending of "stick"

into fish meal is likely to reduce the general protein quality of the latter.

Sample 93, cartilage, gave better results both chemically and biologically than might have been expected. A blend of cartilage with sardine meal (sample 94) was intermediate to these samples in respect to gain. The results with gelatin (sample 95) were similar to those formerly obtained (loc. cit.), while the blend of gelatin with sardine meal (sample 96) gave intermediate growth results in agreement with the calculated index.

Fish meal was used as a standard source of protein rather than the more commonly employed casein. This choice was based on the fact that casein which is deficient in arginine (Klose, Stokstad and Almquist, '38) and in glycine (Almquist, Stokstad, Mecchi and Manning, '40) is an inadequate protein for chicks. In protein test diets, the protein level is usually decidedly sub-optimal in order to insure maximum protein utilization and to insure a rigorous test of the adequacy of the protein supplement. The amino acid deficiencies of casein for chicks in these low-protein diets may produce a situation in which the casein is not a standard protein but, instead, a variable particularly dependent on the amino acid composition of the remainder of the diet.

Due to the interest which has arisen concerning the chemical method for estimating protein quality there have been some attempts to apply the chemical method to products such as vegetable protein concentrates and other types of concentrates which do not properly belong in the group made from animal flesh or skeletal tissues. Such use of the chemical method has not been investigated by us and is not, at present, recommended.

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THE UTILIZATION OF THE CALCIUM OF MILK BY ADULTS

HERTA BREITER, ROSALIND MILLS, JULIA DWIGHT,[1] BEULA McKEY,
WILLIAMINA ARMSTRONG AND JULIA OUTHOUSE

*Department of Home Economics, College of Agriculture, University of Illinois,
Urbana*

INTRODUCTION

In the past, many nutritionists have classified milk as being an almost indispensable component of the American dietary. This high rating has been due primarily to the richness of this food in calcium. But, as has been pointed out before (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39) and so effectively demonstrated in studies on the availability of the calcium of spinach (e.g., Fairbanks and Mitchell, '38) the worth of a food is not determined solely by the amount of a given nutrient contained in it; the extent to which that nutrient can be utilized would also affect the rating of the food. From studies on pre-school children, it is apparent that only about one-fifth of the calcium of milk is available to them (Kinsman et al., '39). However, it is probable that they cannot make any better use of the calcium from other sources—at least, they could utilize the calcium of $CaHPO_4$ to the extent of approximately 20% (Kempster, Breiter, Mills, McKey, Bernds and Outhouse, '40). Comparable data on the adult's ability to utilize calcium are extremely meager; the man studied by Steggerda and Mitchell ('39) is the only adult who has been subjected to an experiment designed specifically to determine utilization. This dearth

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[1] A portion of these data was presented by Miss Julia Dwight in partial fulfillment of the requirements for the degree of Master of Science at the University of Illinois, June, 1940.

of specific information on adults prompted the undertaking of the study reported here, and, since milk rates so highly as a source of calcium, this food was selected for the initial investigation.

<div align="center">EXPERIMENTAL</div>

The general plan of the experiment was similar to that of the earlier calcium studies which have been conducted in these laboratories (Outhouse, Kinsman, Sheldon, Twomey, Smith and Mitchell, '39; Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39), and reference to these publications should be made for detailed information concerning the preparation of the food for serving, the separation of the metabolic materials into their respective periods, etc. An exception to the usual procedure was the institution of 5-day, rather than 7-day, metabolic periods, thus necessitating the use of only five different menus in the basal dietary. Other slight deviations were the change in the quantity of milk calcium—only 175 gm. of coffee cream were allowed for each 5-day period, and the substitution, on 4 of the 5 days, of eggs and bacon or sausage for the breakfast cereal. Moreover, all foods except the vegetables were given in larger quantities. The assortment and quantity of foods are given in table 1. Throughout the experiment, vitamins A and D, in the form of a fortified halibut liver oil, were fed in quantities of, at least, 18,800 and 1880 I.U., respectively, for each 5-day period. In addition, because the dietary was almost devoid of milk, 3 to 6 gm. of a water-alcohol extract of rice-polishings were given daily.[2]

Four women and three men served as subjects for the study. The women, all of whom were members of the home economics staff, probably were considerably better nourished than the men—at least, they had habitually consumed, as milk calcium, the equivalent of 1 pint of milk daily, whereas two of the men

[2] The vitamin A and D preparation was the Hi-vi-tol sold by the E. L. Patch Company, Stoneham, Massachusetts; the rice-polishings extract was the ''Galen-B'' of the Galen Company, Berkeley, California.

TABLE 1

The basal diet used throughout each metabolic period

DAY I Article of food	Weight	DAY II Article of food	Weight	DAY III Article of food	Weight	DAY IV Article of food	Weight	DAY V Article of food	Weight
Breakfast									
	gm.		gm.		gm.		gm.		gm.
Orange juice	100	Orange juice	100	Orange juice	100	Orange juice	100	Orange juice	100
Sausage (pork)	50	Bacon	50	Cereal (cubs)	30	Bacon	50	Bacon	50
Egg	75	Egg	75	Cream	75	Egg	75	Egg	75
Cream (20%)	25	Cream	25	Toast	ad libitum	Cream	25	Cream	25
Toast	ad libitum	Toast	ad libitum	Honey	ad libitum	Toast	ad libitum	Toast	ad libitum
Honey	ad libitum	Honey	ad libitum	Butter	ad libitum	Honey	ad libitum	Honey	ad libitum
Butter	ad libitum	Butter	ad libitum	Coffee	ad libitum	Butter	ad libitum	Butter	ad libitum
Coffee	ad libitum	Coffee	ad libitum			Coffee	ad libitum	Coffee	ad libitum
Luncheon									
Pears	75	Tunafish	120	Liver sausage	2 slices	Sausage	75	Tongue	50
Bananas	75	Lettuce	40	Spiced ham	1 slice	Apples	100	Beets	100
Grapefruit	50	Pickle	15	Pork loaf	1 slice	Tomato juice	100	Lettuce	25
Lettuce	40	Green pepper	15	Lettuce	30	Bread	ad libitum	Prunes	40
Noodle soup	250	Tomato juice	100	Pickle	15	Butter	ad libitum	Bread	ad libitum
Prunes	ad libitum	Prunes	40	Pineapple pulp	80			Butter	ad libitum
Toast	ad libitum	Toast	ad libitum	Pineapple juice	100				
Butter	ad libitum	Butter	ad libitum	Angel food cake	½ cake				
Salad dressing		Salad dressing	ad libitum	Bread	ad libitum				
				Butter	ad libitum				
Dinner									
Beef	150	Ham	150	Chicken	100	Beef	150	Lamb	150
Peas	75	Beans (green)	80	Peas	85	Potatoes	125	Corn	85
Potatoes	125	Sweet potatoes	125	Potatoes	125	Onions	10	Potato	125
Pineapple pulp	80	Apricots	100	Strawberries	75	Beans (green)	80	Strawberries	100
Pineapple juice	100	Toast	ad libitum	Bread	ad libitum	Peaches	80	Biscuits	40
Toast	ad libitum	Butter	ad libitum	Butter	ad libitum	Celery salt	0.5	Butter	ad libitum
Butter	ad libitum	Coffee	ad libitum	Coffee	ad libitum	Toast	ad libitum	Coffee	ad libitum
Coffee	ad libitum					Coffee	ad libitum		

had previously existed on a low calcium dietary for several years. However, all were healthy and apparently normal. Their ages, heights and weights are given in table 2.

The experiment was so planned that the formula for the computation of percentage utilization used in the studies on children (Kinsman et al., '39) could be applied in this study on adults. Calcium was still fed at two levels of intake, but, inasmuch as a maximal value for calcium utilization could not be assured if the adults were in calcium equilibrium, both levels were designed to provide less than the maintenance requirement for calcium and, therefore, to induce negative balances. The lower of these two intakes was provided by a basal dietary; the higher one was derived from the specific food being tested— in this case, milk—which was added to the basal dietary. Obviously, if a smaller calcium deficit existed when this food was being tested, it would follow that the calcium of the test substance was being utilized, and a quantitative measure of its utilization could be secured by applying the following formula:

$$\frac{\text{Net losses during basal period} - \text{net losses during test period}}{\text{Intake during test period} - \text{intake during basal period}} \times 100.$$

The basal series preceded the milk series, and the two were separated by a 12-day interval during which time the subjects were permitted to choose their own dietaries, but large quantities of milk or $CaHPO_4$ had to be a part of it. (This short interval of liberal calcium ingestion was also in effect prior to the basal period.) The basal dietary was fed for 34 days (metabolic periods 8 through 13), and, subsequently, it was supplemented with milk for 24 to 34 additional days (metabolic periods 14 through 19). In both series (i.e., basal and milk), collections of metabolic materials were omitted during the first 4 days.

Pasteurized milk [3] was used throughout the experiment. The quantity which was fed was governed by the need to give

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[3] The milk was obtained from the College of Agriculture herd which included Jersey, Guernsey, Holstein and Brown Swiss cows and had an average calcium content of 121 mg. per 100 gm.

TABLE 2
Daily calcium exchange and the utilization of milk calcium

PERIOD		INTAKE	OUTPUT Urinary	Fecal	BALANCE	INTAKE	OUTPUT Urinary	Fecal	BALANCE
		Subject Jd Age 22 Sex F	Weight in kg. 53 Height in cm. 166			Subject Mh Age 24 Sex M	Weight in kg. 60 Height in cm. 166		
		mg.	*mg.*	*mg*	*mg*	*mg.*	*mg.*	*mg.*	*mg.*
Basal	8	251	178	184	—111	287	110	199	— 22
	9	234	154	128	— 48	274	130	284	—140
	10	250	173	129	— 52	270	124	132	+ 14
	11	245	182	105	— 42	295	150	277	—132
	12	234	182	152	—100	252	120	218	— 86
	13	273	206	116	— 49	267	99	202	— 34
	Ave.	248	179	136	— 67	274	122	219	— 67
Milk	14	462	189	278	— 5	580	118	544	— 82
	15	455	233	228	— 6	593	137	452	+ 4
	16	459	217	326	— 84	597	168	397	+ 32
	17	441	233	232	— 24	569	164	387	+ 18
	18	439	236	266	— 63	560	161	435	— 36
	Ave.	451	221	266	— 36	580	150	443	— 13

% Utilization $\dfrac{67-36}{451-248} \times 100 = 15.3$ % Utilization $\dfrac{67-13}{580-274} \times 100 = 17.6$

PERIOD		INTAKE	OUTPUT Urinary	Fecal	BALANCE	INTAKE	OUTPUT Urinary	Fecal	BALANCE
		Subject Ws Age 22 Sex M	Weight in kg 65 Height in cm. 171			Subject Rd Age 24 Sex M	Weight in kg. 74 Height in cm. 181		
Basal	8	319	144	257	— 82	302	179	321	—197
	9	332	127	304	— 99	291	105	259	— 73
	10	298	139	181	— 22	286	131	313	—158
	11	302	131	247	— 76	273	121	239	— 87
	12	283	111	215	— 43	273	168	284	—179
	13	322	110	276	— 64	308	147	313	—152
	Ave.	309	127	247	— 64	289	142	288	—141
Milk	14	608	146	440	+ 22	906	168	699	+ 39
	15	622	148	464	+ 10	900	174	797	— 71
	16	602	152	496	— 46	879	170	746	— 37
	17	594	133	445	+ 16	828	187	499	+142
	18	589	152	466	— 29	850	197	835	—182
	Ave.	603	146	463	— 5	873	179	716	— 22

% Utilization $\dfrac{64-5}{603-309} \times 100 = 20.1$ % Utilization $\dfrac{141-22}{873-289} \times 100 = 20.4$

PERIOD		INTAKE	OUTPUT Urinary	Fecal	BALANCE	INTAKE	OUTPUT Urinary	Fecal	BALANCE
		Subject Bm Age 22 Sex F	Weight in kg. 55 Height in cm. 167			Subject Hb Age 27 Sex F	Weight in kg. 61 Height in cm. 161		
Basal	8	278	243	147	—112	273	146	180	— 53
	9	273	228	131	— 86	281	134	222	— 75
	10	256	214	123	— 81	268	168	203	—103
	11	244	206	103	— 65	278	121	198	— 41
	12	251	247	95	— 91	271	128	246	—103
	13	280	223	152	— 95	295	147	252	—104
	Ave.	264	227	125	— 88	278	141	217	— 80
Milk	14	622	223	333	+ 66	568	104	452	+ 12
	15	618	253	332	+ 33	582	119	534	— 71
	16	627	290	238	+ 99	568	108	280	+180
	17	573	311	372	—110	564	137	463	— 36
	18	473	283	232	— 42	572	135	330	+107
	19	496	255	255	— 14	589	137	500	— 48
	Ave.	568	269	293	+ 5	574	123	427	+ 24

% Utilization $\dfrac{5-(-88)}{568-264} \times 100 = 30.6$ % Utilization $\dfrac{24-(-80)}{574-278} \times 100 = 35.1$

PERIOD		INTAKE	OUTPUT Urinary	Fecal	BALANCE
		Subject Jo Age 42 Sex F	Weight in kg. 64 Height in cm. 161		
Basal	8	243	156	148	— 61
	9	237	186	164	—113
	10	225	231	150	—157
	11	226	205	109	— 88
	12	219	192	213	—186
	13	240	188	162	—110
	Ave.	231	193	158	—120
Milk	14	501	188	338	— 25
	15	494	201	297	— 4
	16	501	206	363	— 68
	17	497	275	280	— 58
	Ave.	498	218	319	— 39

% Utilization $\dfrac{120-39}{498-231} \times 100 = 30.3$

calcium at a sufficiently low level to insure negative balances—
and thus attain maximal utilization of the milk calcium—and
by the desire to feed almost enough to induce calcium equili-
brium so that, subsequently, the calcium requirements of these
subjects could be determined. Since the amount of calcium
which these subjects lost during the basal period varied
greatly, it is obvious that the amount of milk required would
vary accordingly. The actual amounts fed daily were 180 gm.
for Jd, 220 for Jo, 260 for Hb, Mh and Ws, respectively, and
500 for Rd; subject Bm was given 300 gm. at the beginning of
the experiment, but, when it was found that she was in positive
balance, the quantity was decreased to 200 gm. daily during
the last two periods.

RESULTS

The daily calcium intakes, excretions and balances of the
seven subjects are recorded in table 2. During the 30 con-
secutive days in which the basal dietary was ingested, the
average daily calcium intake was 248, 264, 278, 274, 231, 309
and 289 mg., respectively, for subjects Jd, Bm, Hb, Mh, Jo,
Ws and Rd; the average for the seven subjects was 270 mg.
The corresponding average daily excretions of calcium were
315, 352, 358, 341, 351, 374 and 430 with an average of 360 mg.—
resulting in daily net losses of 67, 88, 80, 67, 120, 64 and 141 mg.
When these values are based on body weight, they represent
intakes of 4.8, 4.8, 4.8, 4.6, 3.6, 4.7 and 3.9 mg. per kilogram
and total calcium excretions of 6.4, 6.4, 6.0, 5.7, 5.5, 5.7 and
5.8 mg. per kilogram.

During the milk series, the average daily total calcium
intakes for subjects Jd, Hb, Mh, Jo, Ws and Rd were 451, 574,
580, 498, 603 and 873 mg., respectively. Of these quantities,
milk contributed 220, 318, 318, 271, 318 and 601 mg. for the
subjects listed in the above order. During the first 4 weeks,
subject Bm's calcium intake averaged 610 mg., over half of
which (361 mg.) was contributed by milk. At these levels
of intake, subjects Jd, Mh, Jo, Ws and Rd were in negative

calcium balance to the extent of 36, 13, 39, 5 and 22 mg., having excreted 487, 593, 537, 609 and 895 mg. calcium, respectively. Subjects Bm and Hb were in positive balance; they stored as a daily average, 22 and 24 mg., respectively. During the last two periods when subject Bm was given only 489 mg. of calcium, she lost an average of 28 mg. of calcium daily. (The calcium intake for subject Hb was not reduced because the alternating positive and negative balances made it impossible to ascertain, until it was too late to change, whether or not the level of intake was suitable.) On the basis of body weight, the calcium intakes were 8.7, 11.1, 9.6, 9.7, 7.8, 9.3 and 11.8 mg. per kilogram; the total calcium excretions were 9.4, 10.7, 9.2, 9.9, 8.3, 9.4 and 12.1 mg. per kilogram.

From the data for these two periods, the following values for the utilization of milk calcium can be obtained for subjects Jd, Hb, Mh, Jo, Ws, and Rd, respectively: 15.3, 35.1, 17.6, 30.3, 20.1 and 20.4%. Two values are computable for subject Bm— 31.8% for the first four periods when she received 300 gm. of milk, and 27.1% for the 10 days on the reduced intake of 200 gm. milk daily; inasmuch as a 10-day period is probably not long enough to insure reliable data, the use of an average for all six periods—i.e., 30.6%—would make for greater accuracy. When this value is included, the average utilization of the entire group of seven subjects becomes 24.2%.

DISCUSSION

The two highest values reported here (i.e., 30.6 and 35.1%) were obtained for the two subjects who were in positive balance; inasmuch as these individuals were ingesting a greater quantity of calcium than was needed for equilibrium, one might raise the question as to whether or not the values represent the maximal ability of these subjects to utilize milk calcium. The question is answered for subject Bm by the fact that, when she was fed a level of calcium low enough to put her in negative balance, her computed rate of utilization was no higher than it had been when she was storing calcium.

One can, therefore, conclude that, even though she was in positive balance, she had been utilizing calcium to her maximal capacity. The same conclusion, possibly, can be drawn for subject Hb whose average calcium intakes and balances approximated, respectively, those recorded for Bm during the basal series and during the first four periods of the milk series. Such positive balances as were observed for these two subjects during the milk series—i.e., 22 and 24 mg.—undoubtedly are indicative of the storage of calcium into tissues which had been depleted during the previous weeks [4] of restricted calcium ingestion. Until the skeletal tissues had been refilled with calcium it is conceivable that calcium utilization would proceed at a maximal rate.

The seven adults of this study fall into two fairly distinct groups—those who utilized 20% or less and those who utilized 30% or more. The cause for such a grouping is difficult to identify, but it is obvious that age differences could not have been responsible. Sex differences also must be ruled out; those subjects showing the most efficient usage of calcium were women, it is true, but the one who utilized the least also was a woman. She had been accustomed to large quantities of milk for many years prior to the experiment, and this fact may have been responsible for her poor utilization of milk calcium, if Rottensten's findings ('38) on rats are applicable to man—i.e., his work would lead one to expect less efficient utilization of calcium in the well-nourished individual and extremely efficient utilization in those whose skeletal tissues had been depleted. However, the three subjects who showed the highest utilization rates were well nourished with respect to calcium. Furthermore, the two who utilized only 17.6 and 20.1% were known to have been on a low calcium dietary for at least 3 years prior to the beginning of the experiment. Obviously, the nutritional status of these subjects could not account for the differences in their capacities to utilize calcium.

[4] Immediately preceding the basal series, these seven individuals had served as subjects in a study for the determination of the utilization of the calcium of carrots; they had ingested, as an average, 446 mg. calcium daily for 39 days.

There are few studies in the literature which yield information concerning the adult's ability to utilize the calcium of milk. Steggerda and Mitchell's experiment ('39) is the only one which actually was designed to give quantitative data; their one adult subject utilized milk calcium to the extent of 20%. However, there are several other subjects mentioned in the literature who had received two different, but moderate, levels of calcium and for whom, consequently, data for utilization can be computed. Two of the presumably healthy subjects studied by Sherman and co-workers utilized milk calcium as follows: a 54 kg. woman, 23% (Sherman and Winters, '18) and subject E, 76% (Sherman, '20). The upper class Indian subject of Basu, Basak and Rai Sircar ('39) utilized 22%, while the four subjects, nos. 4, 5, 6 and 7, of Owen, Irving and Lyall ('40) utilized 71, 31, 51 and 48%, respectively, of milk calcium. Some of these data fall within the range reported in the present paper, but others are considerably in excess; however, the values observed for the subjects of Owen et al. are possibly the result of an adaptation to an extremely inadequate intake of calcium over a period of many years.

It is interesting to note that, whereas the rates of calcium utilization of the eleven children studied in this laboratory (Kinsman et al., '39) fell within the range of those reported for these seven adults, such high utilization as was observed for subjects Bm, Hb and Jo (i.e., 31, 35 and 30%, respectively) was never encountered in any of the children previously studied except during the time of lactose ingestion (Mills, Breiter, Kempster, McKey, Pickens and Outhouse, '40). This disaccharide increased their utilization of calcium 33%, as an average, and the mean of their percentage retentions—i.e., 26.6%—is comparable to the average figure for calcium utilization computed in the present study.

The wide disparity in the values obtained with these adults—and with the children previously studied for utilization of milk

calcium—is good evidence that it is not the form in which calcium occurs in a given food which determines its availability. Additional proof is found in the much more efficient utilization by babies (Wang, Witt and Felcher, '24) and the almost complete usage of the calcium of milk by rats (Ellis and Mitchell, '33). If the state of calcium (i.e., whether or not it is ionized) does determine the rate of utilization, then one would expect all species and all members of a given species to make use of identical portions of the calcium in a specific food. Obviously, the factor which governs the degree of utilization must be situated in the body, but whether it controls the absorption of calcium from the intestines or whether it regulates the deposition of the absorbed calcium into the skeletal tissue is not known. Unfortunately, there is nothing in the data on pathways of excretion (in table 2) which gives any clue concerning the mechanism involved nor the reason why these seven subjects differed in their ability to utilize calcium.

SUMMARY

Seven healthy adults—four women and three men, ranging in age from 21 to 42 years—were subjected to a calcium metabolism experiment for determining their utilization of the calcium of milk. Two different levels of calcium were fed—the lower one, supplied by a basal dietary, averaged 270 mg.; the higher one was obtained by supplementing this basal dietary with enough pasteurized fluid milk to produce a slightly negative balance. The quantity of milk which each subject received was determined by the magnitude of his calcium losses during the basal period; this quantity ranged from 180 to 500 gm. daily. The basal period lasted 34 days, and milk supplemented it an additional 24 to 34 days. On respective, daily, total calcium intakes of 248, 264, 278, 274, 231, 309 and 289 mg., the subjects were in negative balance to the extent of 67, 88, 80, 67, 120, 65 and 141 mg. During the milk period, the calcium intakes were 451, 568, 574, 580, 498, 603

and 873 mg. with resulting balances of —36, +5, +24, —13, —39, —5 and —22 mg. By relating differences in calcium intakes to the differences in the corresponding calcium losses, the following values for the utilization of milk calcium were obtained: 15.3, 30.6, 35.1, 17.6, 30.3, 20.1 and 20.4%. No explanation could be found for the division of the subjects into high and low utilizers.

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FASTING CATABOLISM AND FOOD UTILIZATION OF MAGNESIUM DEFICIENT RATS

MAX KLEIBER, MURIEL D. D. BOELTER AND DAVID M. GREENBERG
*Division of Animal Husbandry, University of California, College of Agriculture,
Davis, and the Division of Biochemistry, University of California
Medical School, Berkeley*

Symptoms and sequelae of magnesium deficiency have been discussed by Kruse, Orent and McCollum ('32, '33), Greenberg and Tufts ('38), Greenberg, Lucia and Tufts ('38), and Tufts and Greenberg ('38). Calcium deprivation also has recently been shown to lead to a definite syndrome, quite different from that due to magnesium deficiency. In some respects calcium and magnesium are physiologically interchangeable, and in others antagonistic. Whereas calcium deficiency leads to lethargy, magnesium deficiency causes tetany. The effects of calcium deficiency upon fasting catabolism, food utilization, chemical composition of the body and size of its various parts have been described in a previous paper (Kleiber, Boelter and Greenberg, '40). A parallel study on magnesium deficiency is reported in this communication.

EXPERIMENTAL METHODS

The basal diet used in this work was identical with that used in the investigation on calcium deficiency (Kleiber, Boelter and Greenberg, '40) except for the salt mixtures.[1]

[1] The quantities of pure vitamins given in table 1 of the publication by Kleiber, Boelter and Greenberg ('40) are per kilogram of food.

The salt mixture used in the magnesium-deficient food consisted of 0.73 gm. NaCl, 1.0 gm. KCl, 2.2 gm. CaHPO$_4$, and 0.32 gm. Fe(NH$_4$)$_2$(SO$_4$)$_2 \cdot$6 H$_2$O per 100 gm. of basal diet, and the control salt mixture differed from this only in the addition of 1.0 gm. of MgSO$_4 \cdot$7 H$_2$O. Chemical analysis showed that the magnesium-deficient diet contained 2.06 mg. and the control 84 mg. magnesium per 100 gm. of food.

The experiments were carried out according to the following plan. Ten rats were placed on the magnesium deficient diet when they were 36 days old. They were allowed an unlimited food intake for the first 5 days, then the animals were paired according to weight and fasting catabolism, and one animal of each pair was continued on the magnesium deficient diet ad libitum, while the pair mate was fed the magnesium supplied control diet in quantities so restricted that its body weight was kept as nearly equal to that of the deficient rat as was possible.

The fasting catabolism was measured twice during the preliminary 5-day period when all rats were on the magnesium deficient food, and three times during the main part of the experiment after the rats had been paired on the deficient and control diets, respectively. The measurement of the energy metabolism was carried out at 30°C. in the same multiple respiration apparatus that was used in the previous trials on calcium deficient rats (Kleiber, '40). The age and body weights of the rats at the time of each respiration trial are given in figure 1 and table 2. At the age of 98 days the rats were sacrificed, the moist and dry weights of their entire body, as well as their hearts, livers, and thyroid and adrenal glands, were measured, and the carcasses were analyzed for fat, nitrogen, ash, and magnesium. The method for the magnesium determination was the same as that used by Tufts and Greenberg ('38).

<center>RESULTS</center>

Growth rate and appetite. The curve for the growth of the magnesium deficient rats, shown in figure 1, is very similar

to that found for the calcium deficient rats. The greater weight attained by the magnesium-deprived animals is readily explained by the fact that the experiment on magnesium deficiency was started on rats that were 10 days older and weighed 35 gm. more than the animals used in the calcium test. The lower curve in figure 1 shows how the appetite was affected by the magnesium deficiency. The decrease in voluntary food intake was decidedly greater and more consistent than was found for calcium deficiency.
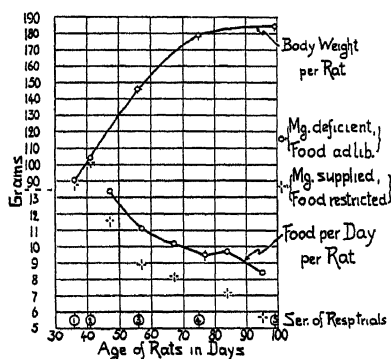
Fig. 1 Growth and food consumption curves of magnesium deficient and control rats, pair fed to maintain equal body weights.

Over the trial period of 57 days the magnesium deficient rats ate an average of 596 gm. of air dry food per animal, while the controls were given an average of only 493 gm. (83% of the food eaten by the magnesium deficient group) in order to maintain equal body weights between pair mates. At the end of the trial, when the rats were 99 days old, the average weight of the deficient rats was 184.4 ± 8.4 gm., and that of the controls 183.8 ± 7.6 gm.

Eppright and Smith ('37) have observed that rats on a control diet gained more weight than the animals fed a "low salt" diet, when both groups were fed rations that provided equal amounts of energy and protein. The results obtained by us with the technique of feeding the amount of food necessary to produce the same gain in weight, rather than giving

equal amounts of food, confirm this observation of Eppright
and Smith of a reduced efficiency in food utilization for growth.

Body composition. Table 1 shows that there was no dif-
ference of great significance in the chemical composition of
the carcasses of the magnesium deficient and control rats with
the exception of the magnesium content which was reduced
to about one-half in the deficient rats. The content of total
body ash, in contrast to the result in calcium deficiency, was
even slightly higher in the magnesium deficient rats. This is in
agreement with the observation of Kruse, McCollum and Orent

TABLE 1

Chemical composition of magnesium-deficient rats and their pair-fed controls

All data represent the mean results for groups of five rats

| | PER RAT | | PER 100 GM. OF MOIST BODY OR TISSUE | |
	Deficient	Control	Deficient	Control
	gm.	*gm.*	%	%
Body weight:				
Moist	184.4±8.4	183.8±7.6		
Dry	67.4±2.4	69.8±5.2	36.6	38.0
Body analysis:				
Ash	7.6±0.4	7.0±0.4	4.1	3.8
Organic matter	59.8±2.0	62.8±4.9	32.5	34.2
Fat (ether extract)	25.2±1.9	27.6±4.2	13.7	15.0
Protein (by difference)	34.6±2.0	35.2±1.6	18.8	19.2
N (Kjeldahl)	5.5±0.3	5.4±0.3	3.0	2.9
Magnesium in the body	0.029	0.055	0.016	0.030
Magnesium in the plasma			0.0013	0.0039
Magnesium in the red corpuscles			0.0028	0.0094
Calcium in the kidney			0.043	0.007

('34) and of Tufts and Greenberg ('38), namely, that there
is a tendency toward increased storage of calcium in mag-
nesium deficiency. Further evidence for the magnesium de-
ficient state of the experimental rats is offered by the
reduction observed in their levels of plasma and red corpuscle
magnesium and the great increase in their kidney calcium,
which are characteristic effects of magnesium deprivation in
the rat (Tufts and Greenberg, '38).

Relative size of parts of body. In contrast to the effect of lack of calcium, magnesium deficiency did not affect skeletal growth. The mean distance from nose to tip of tail was 34.9 ± 0.6 cm. for the magnesium deficient and 35.0 ± 0.1 cm. for the control rats. The mean dry weight of the heart of the deficient animals was slightly greater, but the difference between the weights, 0.153 ± 0.004 gm. and 0.143 ± 0.007 gm., is not statistically significant. The mean dry weight of the liver of the deficient rats, 2.175 ± 0.152 gm., was greater than that of the controls, 1.748 ± 0.128, but the difference is on the borderline of significance, with a random probability of 5.5%. This result parallels our observations on the effect of calcium deficiency as well as those of Eppright and Smith ('37), who found that the weight of the livers of rats on a low salt diet averaged 3.68% of the body weight in mineral-deprived rats as against 2.88% in the controls. The mean dry weight of the thyroid glands was strikingly greater in the magnesium deficient than in the control rats (3.9 ± 0.4 mg. as against only 1.9 ± 0.4 mg.). The dry weight of the adrenal glands was affected in the same way, the mean weights amounting to 23.2 ± 2.3 mg. for the magnesium deficient rats as against only 14.3 ± 1.1 mg. for the controls. No such effect on these endocrine glands was observed in calcium deficiency.

Activity of rats. A. H. Smith[2] tested the activity of the magnesium-deficient rats by the same method which is described in the previous paper, and which indicated a marked decrease in voluntary activity as a result of calcium deficiency. Magnesium deficiency, it was found, did not appreciably affect the activity of the rats.

Fasting catabolism. The results of the five series of respiration trials are summarized in table 2. In the third respiration series, 15 days after the start of paired feeding, the mean catabolic rate of the magnesium deficient rats was already slightly higher than that of the controls although the dif-

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[2] The authors are indebted to A. H. Smith, biochemical assistant in the division of animal husbandry at Davis, for technical assistance in carrying out the respiration trials.

ference was not significant. It became so in the fourth series; and in the fifth series, 58 days after pairing, the catabolic rate was 25% higher in the magnesium deficient rats than in the controls.

This is practically the same increase as was obtained 58 days after pairing with the trials on the calcium deficient rats (series 4, Kleiber, Boelter and Greenberg, '40). Magnesium

TABLE 2

Fasting catabolism of magnesium-deficient and control rats.
Results of five pairs of rats

	SERIES OF RESPIRATION TRIALS				
	1 *	2 *	3	4	5
Age of rats in days:	36	41	56	75	99
Mean body weight in grams:					
Deficient rats	91±4	104±3	146±3	179±4	184±8
Control rats	88±1	101±3	146±2	178±5	184±8
Daily basal catabolic rate in k. cal. per rat:					
Deficient rats	17.8±0.8	20.2±0.7	23.8±1.0	25.6±1.1	26.1±1.4
Control rats	17.3±0.5	19.7±0.9	23.0±1.2	21.7±0.4	20.8±0.5
Catabolic rate per $kg^{3/4}$ † in k. cal.					
Deficient rats	107.6±3.6	109.4±4.7	100.6±4.4	93.2±4.4	93.0±6.6
Control rats	106.6±2.4	109.4±3.7	97.6±5.4	80.0±1.5	74.4±1.4
Ratio of the catabolic rate per $kg^{3/4}$ † of deficient in % of control rats	101	100	103	116	125

* In the first two trials, both groups of rats were maintained on the magnesium-deficient diet.

† Unit of metabolic body size; body weight in kilograms raised to the ¾ power.

and calcium deficiency thus have a very similar effect on catabolism even though many of their other influences on the organism are clearly very different.

Ochoa ('39) has reported that the addition of magnesium to a system consisting of dialyzed brain tissue, fumaric acid, adenylic acid, and cozymase, increased the rate of oxidation. Considering this observation one might expect a priori to find a decrease rather than an increase in the catabolic rate as a result of magnesium deficiency, which, in the present

experiments, reduced the magnesium concentration in the blood to one-third of the normal, as shown in table 1. Tufts and Greenberg ('38) have observed, however, that, even in the last stages of magnesium deficiency, the metabolically active tissues still contain almost normal amounts of magnesium. The magnesium content per unit weight of moist muscle tissue of the deficient rats amounted to 90% of that of the controls. The increase in the catabolic rate observed in magnesium deficiency may thus be related to an increase in the breakdown of tissue because of an inability to secure sufficient magnesium out of intact tissues after the stores in the bones have been depleted. The same reasoning may be applied to calcium deficiency since Boelter and Greenberg ('41) observed that the calcium content per unit weight of muscular tissue of calcium deficient and control rats was essentially the same.

A major difference between calcium and magnesium deficiency with regard to the catabolic relations is the hypertrophy of the thyroid and adrenal glands resulting from magnesium deficiency, but absent in calcium deficiency.

Magnesium deficiency and food utilization. The five magnesium deficient rats gained 80 gm. of body weight per rat in 57 days on 596 gm. of air dry food, while the controls gained 83 gm. per rat on 493 gm., or on 103 gm. less food than their deficient pair mates. This difference of 103 gm. of food represented 530 kilocalories of chemical energy. With this smaller energy intake the control animals gained 2.5 gm. body fat and 0.6 gm. body protein more than the deficient rats, an extra gain of 26 kilocalories. The deficient rats thus wasted 556 kilocalories more energy per animal in the 57 days of trial than did the controls. This extra waste amounts to 9.8 kilocalories per rat per day, or to 18.5% of the mean energy content in the 10.4 gm. of food (53 kilocalories) consumed daily on the average by a deficient rat.

The magnesium supplied controls consumed 19.7 gm. less protein than their deficient pair mates and, moreover, they gained 0.6 gm. more body protein per rat than the deficient

rats. Consequently, the deficient rats wasted 20.3 gm. more protein than the controls in the 57 days, or 0.36 gm. of protein per rat per day. This extra protein waste amounts to 18% of the mean daily intake of 1.99 gm. of food protein. Magnesium deficiency increases the protein waste to about the same extent as it increases the waste of food energy.

The rate of fasting catabolism of the magnesium deficient rats was greater than that of the controls by 5.3 kilocalories per rat per day at the end of the experiment (fifth series) and was considerably less in the earlier series. The increased rate of fasting catabolism, therefore, can account for not more than half of the extra waste of 9.8 kilocalories of food energy per day per rat on the deficient diet.

The decrease in total efficiency of food utilization in magnesium as well as in calcium deficiency results therefore not only from a higher basal metabolic rate but also from a decrease in the partial efficiency of food utilization,[3] namely, a greater loss of unoxidized material in feces and urine, or a higher calorigenic action, or a combination of these two effects.

SUMMARY

1. Five rats fed a magnesium deficient diet gradually lost appetite and, after 60 days on this regime, ceased to grow. Littermate controls could be kept at the same weight as the deficient rats by restricting their intake of magnesium-supplied food to 83% of the consumption of the deficient rats.

2. The magnesium content of the carcasses of the deficient rats was only one-half of that in the control rats, but there was no significant difference between the water, ash, fat, and protein content of magnesium deficient and control rats.

3. Body length was not affected by magnesium deficiency. The dry weights of the heart and liver were slightly greater,

[3] Total efficiency of energy utilization is defined as the quotient
$$\frac{\text{Total gain of body energy}}{\text{Total energy of food consumed}}; \text{ the partial efficiency as the quotient}$$
$$\frac{\text{Change in gain of body energy}}{\text{Corresponding change in energy of food consumed}}.$$

and those of the thyroid and adrenal glands significantly greater in the magnesium deficient animals than in their magnesium supplied controls.

4. The rate of fasting catabolism of the magnesium deficient rats progressively increased in comparison to the rate of the magnesium supplied controls. The catabolic rate of the deficient rats amounted to 125% of the rate of the controls, 57 days after the pairing of the rats.

5. Magnesium deficiency decreased the efficiency of energy and protein utilization to approximately the same extent, causing an extra waste of 18% of the intake. The increased rate of fasting catabolism is not sufficient to explain the extra waste of energy by the magnesium deficient rats. It can therefore be concluded that magnesium deficiency causes an increased loss of unoxidized material in the excreta, or increases the calorigenic action of the food.

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THE RATE OF ABSORPTION OF VARIOUS FATTY ACIDS BY THE RAT [1]

HARRY J. DEUEL, Jr., LOIS HALLMAN AND A. REIFMAN

Department of Biochemistry, The University of Southern California Medical School, Los Angeles

In an earlier report from this laboratory (Deuel and Hallman, '40), it was pointed out that a marked variation obtains in the rate of absorption of the various synthetic triglycerides composed of short-chained fatty acids. Triacetin and tributyrin were the most rapidly absorbed of any of the natural or synthetic fats investigated while tricaproin and tricaprylin were somewhat more slowly utilized. On the other hand, the synthetic fats composed of the odd-chain fatty acids disappeared from the intestine at a rate only approximately 50% of the comparable triglycerides having fatty acids with an even number of carbon atoms. Thus, tripropionin, trivalerin and triheptylin all exhibited a markedly retarded absorption rate.

There are two possibilities which may be advanced to explain this divergence. In the first place it is conceivable that the pancreatic lipase may act preferentially on the even-chain triglycerides, resulting in a much accelerated rate of hydrolysis with these compounds. Since there seems to be general agreement that saponification of the triglyceride molecule must precede absorption, such a variation would offer a satisfactory explanation for the differences in absorption rate we have already noted. However, there are no in vitro experiments which support these contentions; in fact such studies by two groups of workers show widely different

results when the enzymatic hydrolysis is followed under somewhat different conditions (Balls, Matlack and Tucker, '37–'38; Weinstein and Wynne, '35–'36).

A second explanation of the divergent absorption rates of the various synthetic triglycerides may be in the differences in the speed with which the liberated fatty acids leave the gut. That the intestinal mucosa may exert such selectivity in absorption with different fatty acids does not seem difficult to accept since we know that such variations may occur between such closely related hexoses as glucose and mannose (Cori, '25).

In order to test the second possibility we have followed the rate of absorption of the fatty acids from acetic to tridecylic in the fasting rat.

METHODS

In all the tests recorded in table 2 as well as most of the 1-hour tests summarized in table 3, the fatty acids were fed in doses of 100 mg. per 100 sq.cm. of surface area to female rats previously fasted for 48 hours. In the tests with the higher fatty acids the experiments were also carried out for 3-hour intervals in which case the fatty acids were administered at a level of 200 mg. per 100 sq.cm. Surface area was calculated from the fasting body weight by the formula of Lee ('29). We have shown earlier that the most satisfactory basis for comparison of the rate of absorption of fats is on the basis of surface area (Deuel, Hallman and Leonard, '40).

The lower fatty acids (acetic to heptoic) were fed in 20% aqueous solutions as their sodium salts which had been adjusted to a pH of 7.40. Because of difficulty in obtaining sufficiently concentrated solutions of the soaps of the higher fatty acids, they were fed as the free acids. Because of the weakness of these acids, this could be accomplished without any apparent injury to the rats. When the animals were sacrificed at the end of the absorption period, the intestines appeared normal from a gross inspection except in the tests

with caprylic acid where a considerable mucous secretion had occurred in the stomach.

At the conclusion of the absorption tests, the rats were anesthetized with amytal and the intact intestine removed as described earlier (Deuel, Hallman and Quon, '39). For the removal of the fatty acids which were fed as the salts in water solution, the intestines were flushed with 200 cc. of cold water directly into 800 cc. Kjeldahl flasks; 5 cc. of 50% sulfuric acid was added, a spoonful of a talc-pumice mixture and the acids were distilled into an excess of standard sodium hydroxide using an ordinary Kjeldahl apparatus. The distillation was continued until the first appearance of SO_2 fumes was noted in the distilling flask; the flask was immediately disconnected. The amount of excess alkali was determined by titration with acid using phenolphthalein.

In the case of acetic acid it was necessary to modify the procedure by using repeated distillations. To 150 cc. of the gut washings, 5 cc. of 85% phosphoric acid was added, the mixture was boiled until 135 cc. of distillate was collected; 50 cc. of distilled water was added to the distilling flask through an opening in the distilling bulb and 50 cc. was again distilled; this was repeated three or four additional times until the acetic acid was completely distilled. An all-glass distillation apparatus with ground glass connections was employed.

For the recovery of the higher fatty acids which are not distillable with steam, the gut was flushed with 70 cc. of petroleum ether. This procedure was found quite satisfactory for removal of undigested triglycerides and fatty acids in our earlier studies. After drying the petroleum ether extract with anhydrous sodium sulfate, it was filtered into glass stoppered flasks and the residue washed five times with 15 cc. portions of petroleum ether. The washings were added to the original filtrate and the free fatty acid was titrated with standard sodium hydroxide using phenolphthalein after the addition of sufficient isopropyl alcohol to bring about a homogenous mixture with water.

When the intestines were removed immediately after the discharge of known amounts of acids directly into the stomach, and the gastrointestinal contents removed by the procedures outlined above, the recoveries noted in table 1 were obtained. These values have been corrected for the control levels obtained on the intestinal contents of fasted rats which received no fatty acids. Similar corrections are applied to the titrations obtained in the absorption tests (tables 2 and 3).

TABLE 1

The recovery of fatty acids from the gut contents of female rats killed immediately after the administration of a water solution of the sodium salt (I and II) or after the administration of the free fatty acid (III)

FATTY ACID	NO. OF TESTS	BODY WEIGHT	FATTY ACID IN MG.			
			Fed	Recovered		
				Total	Corrected [1]	Per cent [2]
Procedure I (Flushing intestine with 150 cc. H₂O; repeated distillations)						
Acetic	10	114	213	212	203	95.4±0.8
Procedure II (Flushing intestine with 200 cc. H₂O; single distillation)						
Propionic	10	123	239	223	210	87.8±0.8
Butyric	10	128	229	225	210	91.8±0.9
Valeric	10	137	230	217	200	87.0±1.5
Caproic	11	154	258	245	225	87.5±1.2
Heptoic	12	124	226	222	200	88.4±1.5
Procedure III (Flushing intestine with 70 cc. petroleum ether; titration of extract)						
Caprylic	10	86	146	139	137	94.0±1.0
Nonylic	10	75	152	135	133	87.5±1.1
Capric	13	100	182	174	171	93.4±1.0
Undecylic	17	129	215	198	195	91.1±1.2
Lauric	12	126	199	202	199	99.8±1.3
Tridecylic	10	124	192	186	183	95.3±0.9

[1] Based on correction factors for the control fasted rats as follows: Procedure I. An average of ten experiments gave a titration value of 1.59 cc. of 0.1 N NaOH. Procedure II. An average of twenty-two experiments gave a titration value of 1.70 cc. of 0.1 N NaOH. Procedure III. An average of fourteen experiments gave a titration value of 0.16 cc. of 0.1 N NaOH.

[2] Including standard error of mean $= \sqrt{\frac{\Sigma d^2}{n}} \;/\; \sqrt{n}$

d = deviation from mean.

n = number of observations.

TABLE 2

The absorption during a 1-hour period of the distillable fatty acids fed as sodium salts in quantities of 100 mg. per 100 sq. cm. to fasting female rats

FATTY ACID FED	NO. OF EXPTS.	WEIGHT BODY	SURFACE AREA	FATTY ACID IN MG.				
				Fed	Recovered		Absorbed	
					Total	Corrected [1]	Total	Per 100 sq. cm. per hr. [2]
		gm.	*sq. cm.*					
Acetic	10	126	229	229	159	167	62.0	26.6±1.0
Propionic	16	155	257	257	176	200	56.5	21.4±1.2
Butyric	18	155	257	257	142	155	101.9	39.7±1.5
Valeric	11	160	263	253	166	190	62.2	23.3±1.9
Caproic	11	140	241	243	132	150	91.2	38.0±1.7
Heptoic	20	151	252	253	165	187	65.6	25.8±1.5

[1] Correction based on average recovery given in table 1.

[2] Including standing error of mean as in table 1.

TABLE 3

The absorption of the higher fatty acids when fed as such to fasting female rats

FATTY ACID FED	NO. OF EXPTS	LENGTH OF PERIOD	BODY WEIGHT	SURFACE AREA	FATTY ACID IN MG				
					Fed	Recovered		Absorbed	
						Total	Corrected [1]	Total	Per 100 sq. cm. per hr. [2]
		hr.	*gm.*	*sq. cm.*					
Caprylic	23	1	102	199	174	91	97	76	37.3±1.5
	10	3	132	234	469	136	145	324	46.0±1.7
Nonylic	24	1	136	238	240	137	157	83	34.4±2.3
	23	3	146	248	500	222	254	246	32.8±1.3
Capric	22	1	97	193	177	126	140	37	19.2±1.5
	11	3	124	226	452	280	300	153	22.6±2.1
Undecylic	15	1	127	229	229	163	180	49	21.3±3.4
	9	3	126	228	458	284	312	146	21.4±0.5
Lauric	10	1	131	233	232	226	226	6	2.7±1.2
	10	3	126	228	457	429	430	27	3.8±0.7
Tridecylic [3]	15	1	120	221	222	160	175	47	20.8±3.2

[1] Corrected for recovery based on average recovery given in table 1.

[2] Including standard error of mean calculated as in table 1.

[3] Diarrhea occurred in sixteen of eighteen experiments carried out over a 3 hour period. The successful tests were not considered valid.

The fatty acids with the exception of acetic acid were chemically pure products,[2] the purity of which was established by determination of the acid equivalent.

RESULTS

The average results of the absorption tests are summarized in tables 2 and 3. Table 2 records the tests on the distillable fatty acids while table 3 lists the experiments on the higher fatty acids. The statistical evaluation of the rates of absorption of acetic, butyric, caproic, and caprylic acids when compared with the corresponding odd-chain fatty acids is given in table 4 on the basis of the Fisher "t" method ('34).

TABLE 4

The statistical evaluation of mean differences based on the Fisher "t" method

FATTY ACID	NEXT LOWER ACID		NEXT HIGHER ACID	
	"t" calculated	Significant value for "t"[1]	"t" calculated	Significant value for "t"[1]
Acetic	....	....	2.83	2.78
Butyric	9.19	2.75	6.57	2.76
Caproic	5.47	2.82	4.98	2.75
Caprylic (1 hr.)	5.23	2.75	1.01	2.75
Caprylic (3 hr.)	....	....	5.59	2.75

[1] Based on a "P" value of 0.01 (chance of difference in results being due to error in sampling 1 in 100).

DISCUSSION

The more rapid rate of absorption which we have noted earlier for the triglycerides composed of the short-chain fatty acids with an even number of carbon atoms as contrasted with those having an odd number of carbons apparently is related to a difference in the speed of absorption of the fatty acids themselves. Thus, the rates found for the absorption of acetic, butyric, caproic and caprylic acids are in each case considerably higher than those for the corresponding odd-carbon acids. Such differences are in every case highly signifi-

[2] The lauric acid was kindly furnished by Dr. Samuel Lepkovsky, of the Eastman Kodak Co. Glacial acetic acid of Baker and Adamson was used.

cant when based on the Fisher "t" method (table 4) except in the comparison of caprylic and nonylic acids. Although the differences in rate observed in the latter case are not significant in the 1-hour tests, the variation in the experiments which lasted 3 hours are highly significant.

The rate of absorption of the distillable fatty acids under the present experimental conditions is somewhat less than that observed earlier for the corresponding triglycerides but the proportionate rate is similar except with sodium acetate. This difference would seem to be traceable either to the smaller dose employed (100 mg. instead of 300 mg. per 100 sq.cm.) or to the variable effect of water solution as compared with the fat itself. The presence of the sodium ion may alter to some extent the permeability of the intestinal mucosa. Although the results with sodium acetate are significantly higher than those found with sodium propionate, they show a much slower rate than was expected from the experiments with triacetin where the most rapid absorption rate obtains. This may possibly be due to the irritant action of the sodium acetate which tends to prevent its absorption because we have found with dogs that it may act as a violent cathartic (Deuel and Milhorat, '28). However, such catharsis was not observed in the present tests.

Capric, undecylic and tridecylic acids are absorbed at a uniform rate of approximately 20 mg. per 100 sq.cm. per hour. However, they disappear at a rate considerably below that for caprylic acid and somewhat below that of nonylic acid. Essentially similar results were noted in the 1- and 3-hour tests which would indicate a constancy in the rate of absorption.

It is also significant that the lower rate in absorption obtains with those fatty acids which are insoluble in water. Because of their insolubility, the rate of formation of the fatty acid-bile salt complex may be a limiting factor in determining the speed of absorption; on the other hand with the shorter chain acids which are soluble in water, absorption should not require the formation of this complex.

The higher acids which show the lower rate of absorption are also those which give typical soaps on neutralization with alkali. However, unpublished results [3] indicate that only minimum amounts of the hydrolyzed products may be in the form of soaps. After the administration of hydrogenated cottonseed oil, the pH of the intestine was found to be approximately 7.0. This would be impossible if soap were present in large amounts since it can be buffered only by the fatty acid which is present in solution.

On the other hand lauric acid is absorbed very slowly; less than 5% (27 mg.) of that administered left the gastrointestinal tract over a 3-hour period. The slow rate of absorption may be related to the fact that it is solid at body temperature. At the termination of the absorption test, the acid which was fed in the melted state was found to have solidified in the stomach. On the other hand, trilaurin which melts at a temperature approximately 3° higher, was absorbed to the extent of 216 mg. in a 3-hour test or 22% of that fed. Although this was the lowest value noted for any triglyceride studied, it is more than five times the rate which we have found for the free acid. One would expect, however, that the lauric acid when fed as a component of a natural fat would be much more rapidly absorbed. Here the separation of solid acid in the stomach would be avoided either by solution in other triglycerides which are liquid at body temperature or because it exists as a component of a mixed triglyceride.

SUMMARY

A comparison has been made in the rate of absorption of the fatty acids by the rat from acetic to tridecylic acids inclusive. The distillable fatty acids (acetic to heptylic) were fed as the sodium salts while the higher homologues were administered as the free acids.

[3] Experiments of J. W. Mehl, L. F. Hallman and H. J. Deuel, Jr.

Butyric, caproic, and caprylic acids were absorbed the most rapidly. Propionic, valeric and heptoic acids disappeared from the intestine much more slowly while nonylic acid occupied an intermediate position. The wide discrepancy which we have noted earlier in the rate of absorption of the triglycerides composed of even- and odd-chain fatty acids may be explained on the basis of differences in removal of the component fatty acids from the gut.

The absorption of sodium acetate was much slower than might be expected from the earlier results on triacetin although the rate was greater than for sodium propionate. Capric, undecylic and tridecylic acids were absorbed at rates approximating 20 mg. per 100 sq.cm. per hour as determined in experiments of 1 and 3 hours in duration. Only minimal quantities of lauric acid left the gastrointestinal tract over a 3-hour period. The slow speed of absorption may be partly ascribed to its solidification in the stomach; however, trilaurin which has a higher melting point was shown earlier to be absorbed much more rapidly.

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BASAL METABOLISM AND HEAT LOSS OF YOUNG WOMEN AT TEMPERATURES FROM 22°C. TO 35°C.

CLINICAL CALORIMETRY NO. 54

JAMES D. HARDY, ADE T. MILHORAT AND EUGENE F. DU BOIS

WITH THE TECHNICAL ASSISTANCE OF G. F. SODERSTROM

Russell Sage Institute of Pathology in affiliation with the New York Hospital and Department of Medicine, Cornell University Medical College, New York

FIVE FIGURES

INTRODUCTION

In 1939 Hardy and Milhorat published a preliminary report of calorimeter studies on three normal women showing that these women responded to warm environments by a drop in basal metabolism that had not been found in the two men studied under similar conditions. They called attention also to several other differences in heat loss, skin temperature, vaporization, and the conduction of heat through the skin. The results of observations on seven women were discussed briefly by Hardy and Du Bois ('40). This present report contains the complete calorimetric study of eight female subjects and discusses the points of difference between men and women.

Max Rubner in 1890 and 1902 described two forms of temperature regulation, (1) a "physical regulation" through increased vaporization in the warm zone and shivering or other forms of activity in the cold zone, and (2) a "chemical regulation" which changes heat production without muscular movement (or activity of the sweat glands). There seems to be no

doubt that chemical regulation exists in animals and it is well discussed by Giaga ('38). F. G. Benedict ('38) found that the "zone of thermic neutrality," the zone of lowest basal metabolism, was narrow in the case of the dog, pigeon, and mouse, and extremely wide in the case of the goose, steer, cow and sheep. In most species this zone is not far from 28°C. There has been no satisfactory evidence as to the nature of the substance or mechanism which causes a high basal metabolism in the cold and very warm zones, or, perhaps we should say, the low metabolism in the neutral zone. In the cold zone there may, of course, be some slight shivering or tension of muscles that cannot be detected by ordinary means. Burton and Bronk ('37) have described an electrical method of detecting the onset of shivering in animals which should be of use in future studies of this type. There is as yet no real evidence of "chemical regulation" in men. Hardy and Milhorat apparently were the first to demonstrate that it exists in women.

There have been several reports of slight differences in metabolism with the season of the year but there are many influences at play and the results are conflicting. Martin ('30) in a careful study of his own basal metabolism during a voyage from England to India noted a drop of 12% a few days after entering the Red Sea and a return to the previous level when the weather was cooler. Eleanor Mason ('34) who studied a group of women in India and again in England or the United States noted that most of them gave results about 5% higher in the temperate zone. In both of these cases there was time for a slow adjustment and perhaps changes in the food. In our studies the period of adaptation was only 2 to 3 hours.

There have been a good many studies (McConnell, Yaglou and Fulton, '24) of the effects of environmental temperature on the metabolism of men, but very few on women (Hick, Keaton, Glickman and Wall, '39). Many of the investigations have been performed when the metabolism was so far above basal that many of the physiological reactions were obscured.

Wiley and Newburgh ('31) gave the average curve of a large number of experiments on one normal man. When nude there was little or no change in the basal metabolism from about 25° to 36°C.; when clothed the range of level metabolism was from 18° to 34°C. Hardy and Du Bois ('38 a) using the respiration calorimeter of the Russell Sage Institute of Pathology found a level basal metabolism in two normal men from 23° to 34.5°C. The experimental conditions used in that study were duplicated for this present report on women. Quite recently Albagli ('39) has published in Brazil an excellent monograph reviewing the influence of climate on basal metabolism, expressing the belief that most of the differences which have previously been ascribed to climate and race are due to differences in nutrition. He publishes a series of studies on thirteen young men exposed to temperatures between 14° and 39°C. Most of the naked men showed little change in metabolism between 23° and 34°C. The clothed men had a level zone between about 18° and 34°C. If his results are plotted there is a slight indication of a minimal metabolism between 24° and 32°C. and a rise of 2 or 3% between 22° and 24°C., and 32° and 34°C.

Winslow, Herrington and Gagge ('37) have performed many careful experiments on young men sitting in a chair after food had been ingested, and observed an average metabolism about 33% above basal. The men when naked showed slight change in heat production over a range of 20° to 39°C. "operative temperature," an index which takes into account not only the temperature of the air but also the radiant temperature of the walls. Their normal men, lightly clothed, had a level metabolism between 20° and 39°C. In general, the literature indicates that men show an almost uniform level of metabolism at air temperatures between 23° and 34°C.

It has long been known that women have a lower basal metabolism than men but the estimates as to the average difference have ranged from 7 to 15%. Perhaps this comparison is due to the fact that most of the tests on women have been performed at a temperature somewhere in the range of falling

metabolism demonstrated by Hardy and Milhorat. Others have surmised differences between the sexes in thermal response. Oppel and Hardy ('37) could find no differences in their studies of the thermal sensitivity of the skin.

METHOD

The experimental method was essentially that used for the study of the male subjects and has been described in detail in previous publications (Hardy and Du Bois, '38 b). Some additional procedure was desirable in the present study and the routine of an experiment is briefly described. The subjects were selected after a general physical examination. Dates of menstruation were recorded although experiments were carried out regardless of this factor, excepting the first day of menstrual flow. The subject arrived at the laboratory in the postabsorptive state, having taken special care to do little or no walking in transit to the laboratory. She sat quietly in indoor clothing in the calorimeter room, which had been set to the experimental temperature the day before. This period lasted for at least 1½ hours before the beginning of the experiment. The nurse was then called and assisted in taking surface temperature measurements as the subject undressed. The nurse also weighed the subject, adjusted the rectal thermometer, and placed the subject in the calorimeter. (In this connection the authors wish to express their appreciation of the enthusiastic cooperation of Miss R. H. Roberts and her nursing staff.) Following the sealing of the calorimeter there was a preliminary period of an hour, at the end of which the surface temperature was measured by the subject. The next period was used to measure the basal metabolism and after the end of the period the surface temperature was again measured. The mean of these values was considered the average skin temperature during the basal period. There followed either another basal period or a period during which some agent (such as chill, alcohol, exercise, etc.) was studied. At the end of the experimental period the subject's blood pressure was measured.

Eight subjects have been investigated. They were all highly intelligent and carried through the simple laboratory routine without difficulty. Information in regard to diet, etc., could in all cases be depended upon. Body types represented by this group of subjects were essentially normal but included tall and slender as well as short and heavy builds.

<div align="center">RESULTS</div>

The experimental data on three individual subjects are recorded in table 1. The experiments are listed in order of increasing calorimeter temperature, and in the first part of the table are recorded the data relating to heat production. In the latter part are the data relating to heat loss.

1. Basal metabolism. At least 1 and often 2 hours of the experimental period were used for measurements of heat production in the basal state. Care was taken at the colder temperatures that no shivering or tenseness from chilling occurred during these periods. With subject no. 6, it was not possible to obtain an experimental period completely free from tenseness and slight shivering except in the very warm temperatures. In all warm experiments it was necessary to end the period between bursts of sweat rather than at a specified time as the slightest movement of the subject was sufficient to raise the pressure in the calorimeter above that of the barometer. The irregularity of vaporization made it difficult to obtain good respiratory quotients at high temperatures. So far as could be judged from observation and from talking with the subject, the periods specified as basal were intervals during which the subject lay on her back motionless but awake.

The changes in basal metabolic rate for the group are shown in figure 1. In contrast to the male subjects previously studied, whose basal metabolic rates were constant throughout this range of temperature, the women, with the exception of no. 3, all showed a definite change in their basal metabolism in the warmer environments. For temperatures between 22°C. and 27°C. the average level of metabolism was fairly constant (between 31 and 38 cal./hr./M²); between 27°C. and 32°C.

Representative data obtained from three women lying nude under basal conditions in the calorimeter

SUBJECT; DATE; REMARKS	END OF PERIOD	CO₂ gm.	O₂ gm.	RQ	H₂O gm.	URINARY N PER HOUR	HEAT PRODUCED	CALORIMETER TEMPERATURE	% RELATIVE HUMIDITY	RECTAL TEMPERATURE	HEAT ELIMINATED	SKIN TEMPERATURE Time	°C
No. 1 Feb. 2, 1938 Period I Basal	10:33 11:37	23.0	19.8	0.86	27.1	0.53	66.1	23.14	23	37.22 37.00	87.8	9:45 10:20 11:40	30.4 30.7 30.3
No. 1 Feb. 9, 1938 Period I Basal	10:45 11:35	18.1	15.4	0.87	23.3	0.42	51.8	23.27	25	37.40 37.19	69.1	9:55 10:31 11:42	31.9 31.6 30.6
No. 1 Jan. 19, 1938 Period I Basal	10:54 11:54	20.3	18.0	0.83	24.8	0.54	59.8	25.20	20	37.18 37.02	72.2	9:50 10:40 12:00	32.4 31.6 31.5
No. 1 Jan. 12, 1938 Period I Basal	11:20 12:20	20.9	18.5	0.83	28.9	0.42	61.8	26.88	20	37.35 37.30	66.5	10:10 11:00 12:25	32.9 32.5 32.3
No. 1 Mar. 11, 1938 Period I Basal	10:42 11:42	18.5	15.7	0.87	28.7	0.36	53.0	30.30	20	36.97 36.96	53.8	9:45 10:30 11:45	33.6 34.3 34.4
No. 1 Mar. 2, 1938 Period I Basal Period II Basal	10:53 11:53 12:53	19.8 20.7	16.8 20.2	0.87 0.74	38.8 56.3	0.35	56.5 66.2	33.70 33.77		37.50 37.61 37.73	47.5 56.0	10:00 10:35 12:00 1:00	34.6 35.7 36.1 35.6
No. 2 Mar. 25, 1938 Period I Basal	11:12 11:32	6.9	4.1	1.33	6.2	0.27	14.1	23.98	20	36.94 36.95	22.4	10:50 11:35 11:45	30.8 30.6 30.7
No. 2 Jan. 21, 1938 Period I Basal	11:20 12:20	19.0	16.7	0.83	23.4	0.29	55.8	24.87	21	36.94 36.75	66.7	9:50 10:30 11:05	32.5 32.1 31.6
No. 2 Jan. 14, 1938 Period I Basal	10:52 11:52	18.9	16.7	0.83	26.1	0.31	55.9	26.81	20	37.27 37.14	63.1	9:45 10:30 12:10	32.5 32.6 32.1
No. 2 Feb. 16, 1938 Period I Basal	10:30 11:30	18.8	15.6	0.89	26.5	0.28	52.8	28.17	20	36.98 36.94	52.1	9:40 10:19 11:34	31.7 32.4 32.6
No. 2 Feb. 4, 1938 Period I Basal Period III Basal	10:40 11:40 12:40	17.2 18.6	15.2 17.5	0.83 0.77	26.3 25.7	0.38	50.6 57.8	28.21 28.38	20 16	37.16 36.96 37.00	53.4 55.1	10:30 11:45 12:45	33.0** 32.8 32.6
No. 2 Feb. 18, 1938 Period I Basal	10:45 11:45	15.8	13.3	0.87	23.7	0.24	45.0	29.82	19	36.86 36.80	47.9	9:52 10:32 11:51	33.0 34.0 33.8

No.	Date / Period	Time									Time	Temp
No. 2	Mar. 18, 1938 Period I Basal Period II Basal	10:49 11:49 12:49	16.3 16.2	12.8 13.8	0.96 0.87	29.0 47.3	0.30	43.8 46.5	31.13 31.25	20 17	36.74 36.70 36.71	42.8 56.1	10:35 11:55 12:54	34.3 34.6 34.6
No. 2	Feb. 25, 1938 Period I Basal	10:42 11:42	16.6	13.5	0.91	28.9	0.29	45.9	32.02	17	36.80 36.80	41.6	9:45 10:30 11:45	34.4 34.9 35.0
No. 2	Mar. 4, 1938 Period I Basal	10:48 12:00	18.9	16.5	0.84	69.2	0.25	55.4	35.52		37.07 37.21	52.4	9:50 10:35 12:05	33.7 35.8 35.7
No. 2	Mar. 9, 1938 Period I Basal Period II Basal	10:48 11:48 12:49	16.8 18.8	16.1 15.5	0.75 0.89	81.2 88.3	0.18	53.2 52.8	35.89 35.75	26 30	37.23 37.30 37.30	52.1 55.2	9:45 10:29 11:55 12:53	34.2 35.8 35.5 35.8
No. 3	Mar. 14, 1939 Period I Basal; cold at end	10:45 11:41	16.7	14.0	0.87	24.1	0.44	47.1	24.59	26	36.77 36.66	67.7	9:43 10:34 11:45	32.4 31.6 30.5
No. 3	Mar. 7, 1939 Period I Basal	10:40 11:40	17.9	15.5	0.84	23.8	0.67	51.2	25.98	24	36.61 36.57	65.0	9:45 10:26 11:47	32.1 32.3 31.8
No. 3	Feb. 28, 1939 Period I Basal	10:32 11:12	11.8	9.2	0.94	17.9	0.72	30.8	26.00	28	36.83 36.71	44.9	9:33 10:16 11:17	33.6 32.3 31.8
No. 3	Jan. 24, 1939 Period I Basal	10:30 11:30	18.6	15.9	0.85	32.8	0.65	52.8	27.97	25	36.81 36.88	55.5	10:13 11:38	32.4 32.7
No. 3	Feb. 7, 1939 Period I Basal	10:36 11:36	17.5	14.7	0.87	33.6	0.28	49.6	29.84	25		50.0	9:31 10:19 11:42	33.4 33.5 34.1
No. 3	Feb. 14, 1939 Period I Basal	10:19 11:19	17.5	15.5	0.82	35.7	0.35	51.7	31.85	25	37.12 37.03	48.2	9:26 10:08 11:26	34.4 35.1 35.2
No. 3	Feb. 21, 1939 Period I Basal Period II Basal	10:25 11:25 12:25	18.4 17.3	13.6 16.1	0.98 0.78	64.4 66.4	0.55	46.1 52.7	33.98 33.88	33 29	37.24 37.19 37.18	49.9 50.3	9:24 10:05 12:29	35.0 35.2 35.2

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the metabolic rates of all subjects, excepting no. 3, decreased to an average of 30.9 cal./hr./M². In table 2 are given the

TABLE 2

Summary of data on basal metabolism of seven women studied in the calorimeter of the Russell Sage Institute of Pathology

SUBJECT	SYM-BOL	AGE	HEIGHT	WEIGHT	AVERAGE LEVEL OF METABO-LISM AT TEMPERA-TURES BE-LOW 27° C.	TEMP OF LOWEST METABO-LISM	AVERAGE LOWEST METABO-LISM	% DE-CREASE
			cm.	kg.	cal./M²/hr.	°C.	cal./M²/hr.	
No. 1	●	35	175	64	35.6	30.5	30.6	14
No. 2	○	25	162	60	35.8	32	28.2	21
No. 3	×	25	165	55	33.1	32	32.8	1
No. 4	◕	21	162	77	33.6	30.5	29.0	14
No. 5	⊙	23	168	56	35.0	28	32.0	9
No. 6	△	42	169	53	36.2	32.4	33.0	9
No. 7	+	26	165	54	36.0	32	31.0	14
				Averages	34.9		30.9	12
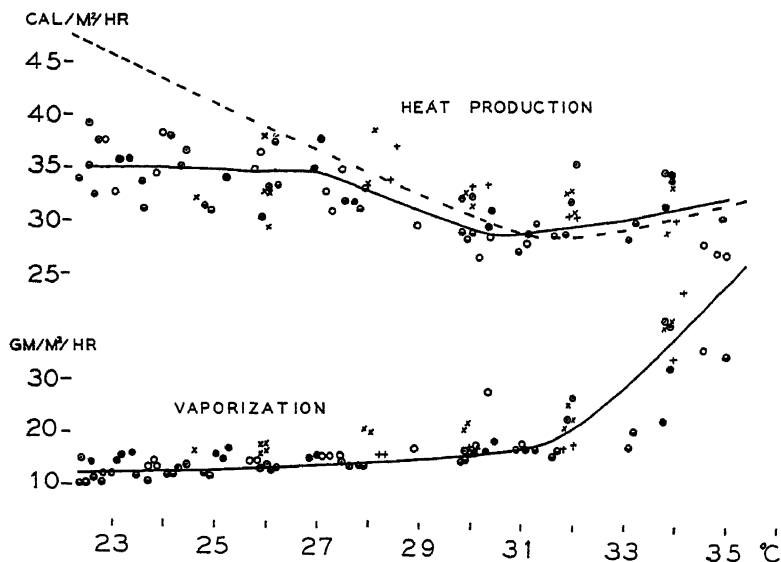
Fig. 1   Upper curve, average basal metabolic rate plotted as a function of calorimeter temperature. Dashed line, average heat loss. Lower curve, average loss of moisture from skin and lungs as affected by calorimeter temperature.

values for the individual subjects. It may be coincidence that the only subject who failed to show at least a 9% fall in metabolism was the one who was receiving hormone therapy. Further study would be needed to establish any such relationship between change in basal metabolic rate and hormone activity.

The change in basal metabolism with environmental temperature observed with these women subjects is the first indication of a "chemical regulation" of body temperature we have been able to find in our studies covering two men and seven women. The level of basal metabolism used for clinical purposes is 36.5 to 37.0 cal./hr./M^2 for women 20 to 35 years of age. In the cold zone, our group, on this basis, has a basal metabolic rate of —5%. Common biologic practice is that of using the metabolic rate in the zone of "thermic neutrality" as the standard of reference. This usage would place our group 19% below the accepted standards. Possibly some allowance should be made in clinical practice for the metabolic adjustment of women to environmental temperature.

2. Water loss from vaporization. In figure 1 are plotted the data for vaporization from skin and lungs. Below 32° C. there was no sweating (except possibly in the axillae) and the water loss was therefore by insensible perspiration. There was a gradual decrease in the water loss as the temperature was lowered from 32° C. to 22° C. This may be due to a drying out of the superficial tissues accompanying the more or less complete vasoconstriction at these temperatures. Above 32° C. sensible sweating was present in nearly all subjects although the amount of sweating was dependent in great part on the amount of activity of the subjects.

3. Rectal temperature. The rectal temperatures of the subjects as affected by environmental temperature are shown in figure 2. The values were higher on the average in the warmer zone than in the cold zone, although the rectal temperatures appeared lowest at 31° C. calorimeter temperature, the point of lowest metabolism. There was a spread of more than 0.6° C.

in the values throughout the whole temperature range, possibly the result of the oestrous cycle or other factor.

4. *Average skin temperature.* In environments colder than 33°C. the average skin temperature fell almost linearly with surrounding temperature. Above 33°C. the highest observed
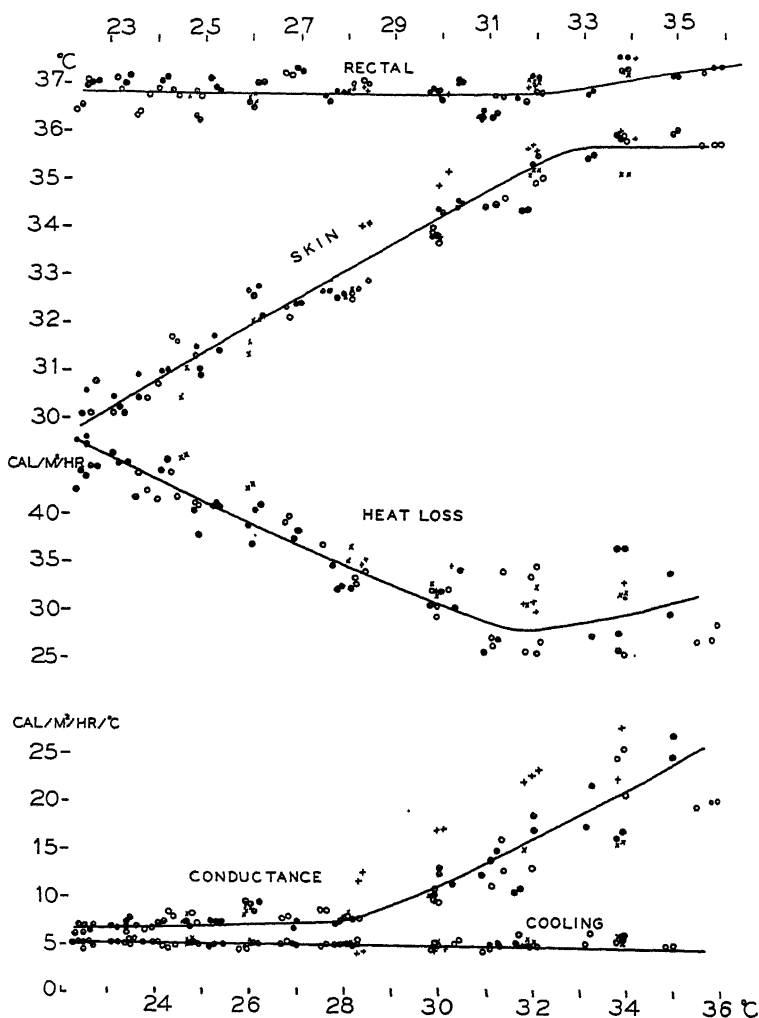
Fig. 2   Relation of calorimeter temperature to rectal temperature, skin temperature, heat loss, peripheral conductance, and rate of cooling from the skin.

values of average skin temperature were about 36°C., and the spread in the values was about 1.2°C., or about twice that for the rectal temperature.

The change in average surface temperature was +0.57°C. per degree change in calorimeter temperature. This is nearly 0.1°C. per degree greater than that observed for our male subjects under like conditions. The fact that some of the subjects had more or less constant skin temperature in the hot experiments while others continued to rise is the counterpart of the variable findings in regard to sweating. Winslow, Herrington and Gagge ('38) have pointed out that in the physiological range of environmental conditions, the body vaporizes exactly the amount of water required to eliminate the heat. Thus, sweating and surface temperature responses can be looked upon as interdependent factors in this zone.

5. Heat loss. Figure 2 shows the values of heat loss for all subjects plotted through the experimental temperature range. The responses to temperature for the subjects show some individual characteristics and the curve represents the average response. This average has a low value of 28.5 cal./hr./M² at 32°C. and increases to either side. In the colder zone the heat loss increases at the rate of 2.3 cal./hr./M² for each degree drop in calorimeter temperature. Above 32°C. there is a considerable spreading of the heat loss values although there is a definite tendency to increase with temperature. This spreading would be expected on the basis that the subjects did not all have the same heat production at the higher temperature; for each subject the heat loss and heat production were nearly the same in the warm experiments. This spread represents a real difference in the response of individual subjects to warm atmospheric conditions.

It is felt that the low values of heat loss at temperatures of 30°C. to 33°C. lend significance to the low values found for heat production. That is, the body was in thermal equilibrium during these changes, and decreased heat loss, caused by higher environmental temperature, was met by decreased metabolism.

Aside from the evident variations in individual response in the hot zone it is of interest to note that in the cold zone the heat loss level for the thin subject was above the average, whereas that for the stout subject was low. As a result of less peripheral tissue, no. 3 had higher conductance than no. 4 and higher rate of vaporization; these two factors account for the differences in their heat loss.

6. Peripheral conductance. The thermal conductance of the superficial tissues can be calculated from the heat loss and the skin and rectal temperatures. Its importance as an index of effective vasomotor action in the periphery has been discussed (Hardy and Soderstrom, '38). Below 28°C. the conductance decreases slowly as the temperature falls. In this temperature range the level and rate of change of conductance were approximately the same for all subjects. Above 28°C. the conductance rose rapidly indicating superficial dilatation. In this temperature range two rates of increase were observed; the lower rate (the same as observed in the men subjects) was 1.7 cal./M²/hr./°C. per degree rise in calorimeter temperature; the higher rate was 3.3 cal./M²/hr./°C. per degree. Number 7 who complained of difficulty in warm atmospheres showed an abnormally high conductance. The conductance was so high that the vessels became engorged and an edema resulted. This was her complaint in summer weather.

It is generally accepted that the change in conductance in warm atmospheres is brought about by control of blood flow in the superficial tissues. Hardy and Soderstrom ('38) point out that the conductance in the cold zone is constant for the men studied in the calorimeter. With the women the conductance decreased 1.56 cal./M²/hr./°C. when the calorimeter temperature changed from 28°C. to 22°C. This is a significant change and may be due to a slow vasoconstriction of deeper vessels or to the gradual cooling of the body. In any case, this change in conductance represents a deeper penetration of the thermal gradient into the body. Thus, at 22°C. the average depth of the gradient is estimated at 24 mm. and at 28°C. the depth is 19 mm. Thus, below 28°C. one may have a vasomotor

effect extending to the deeper vessels whereas in the warmer atmospheres the activity is in the ·superficial vessels. A most important adjustment to environmental temperature change in the warm zone is accomplished by the changes in blood flow in the peripheral vessels, but little protection against cold is offered by the conductance change below 28°C.

No explanation is at hand to account for the two separate responses above 28°C. It is possible that this is an individual reaction depending upon acclimatization, etc. The one fact which is common to all subjects is that within our experimental temperature range, the conductance above 28°C. increases in direct proportion to the environmental temperature.

7. Newton's law of cooling. A check on the overall calorimetric and thermometric techniques is the value of K in Newton's cooling law. The value for the women subjects was 5.3 ± 0.4, within the experimental range. The largest deviations were at the higher temperatures as would be expected. This was the same value as found for the men and means only that the thermal heat loss per degree (the total heat loss minus the loss due to vaporization) was the same for both sexes. This was to be expected as the general configurations of the subjects were the same.

8. The average body temperature. Much discussion has centered around the proper formula for measuring the heat lost or stored in the body by means of changes in rectal temperature and average skin temperature. Burton ('35) proposed the formula $0.67 R + 0.33 S$ (R = rectal temperature; S = average skin temperature). Hardy and Du Bois found the formula $0.8 R + 0.2 S$ best fitted their observations on men. Winslow, Herrington and Gagge found Burton's formula more valid for their observations for colder environments. In order to test the matter further, the fifty observations on the women subjects during basal periods have been analyzed. In figure 3 are plotted all the data for observed and estimated changes in body temperature. The observed change in body temperature was computed using 0.83 as the average specific heat of the body.

In the upper left of figure 3 are plotted the differences in the estimated and observed changes in body temperature as a function of the calorimeter temperature; that is, the estimated temperature change minus the observed temperature change is plotted as ordinate and calorimeter temperatures as abscissae. The spread of the values is shown in the small
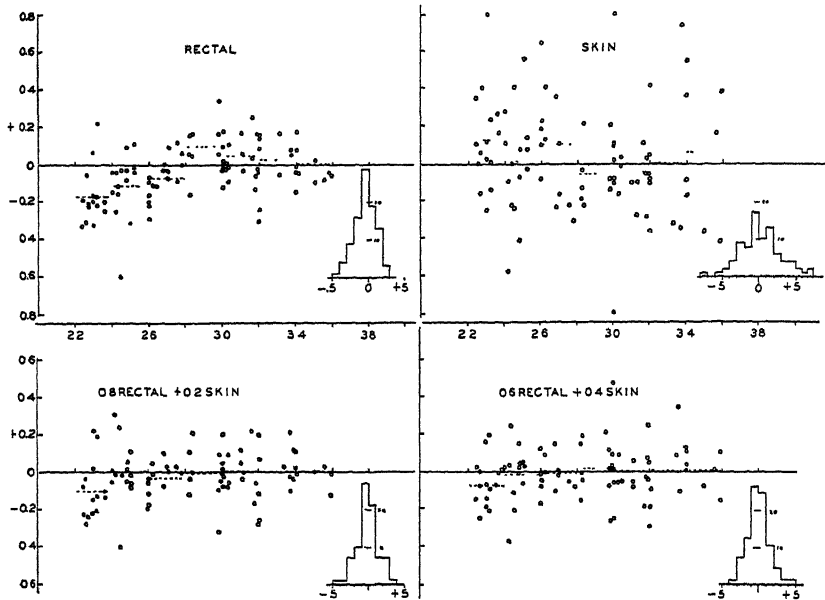
Fig. 3   Graphic analysis of change in average body temperature estimated in four ways as affected by calorimeter temperature. Abscissae calorimeter temperature; ordinates difference in the actual change in body temperature and the estimated change; each point represents change during an hour. Small histograms represent spread of estimated values about the true value. Abscissae degrees Centigrade. Ordinates number of measurements.

histograms. It is evident that in the colder atmospheres the rectal temperature change failed to give a true index of body temperature change, as the body cooled much more than the rectal temperature showed. This difference was less as the calorimeter temperature increased, and above 30°C. the change in rectal temperature on the average was a good index of body temperature change. Of the ninety-four measurements shown,

seventy-seven of them gave the true change in body temperature within $\pm 0.2°$C.

The upper right of the figure shows the skin temperature as an index of body temperature. At low temperatures the skin cooled more than the body, and above 28°C. the skin temperature served as a fair index of body change. The spread of the values of body temperature calculated from skin temperature was large, only forty-eight of the eighty-six readings falling within $\pm 0.2°$C. of the actual change.

That the skin temperature overshoots the mark and the rectal temperature undershoots it lends confidence to the hope that a combination of the two will give a fairly accurate estimate of the body temperature change. The lower left shows the estimation by the formula suggested by Hardy and Du Bois and the lower right the estimation approximating that of Burton. The temperature range from 22°C. to 24°C. shows both formulae to fail, the Hardy and Du Bois formula by — 0.10°C. and the Burton formula by — 0.06°C. From 26°C. on up either formula works, with slightly better estimates obtained by the Burton method. It is evident that a much greater weight than 40% must be given to the skin when temperatures below 24°C. are reached. It is not surprising that no single formula can be used for the whole temperature range or for all subjects. The women, having more subcutaneous fat would be expected to differ slightly from men toward greater weighting of the skin temperature. Above 24°C. good estimates of change in body temperature may be obtained with either 80% R + 20% S or 60% R + 40% S. Below 24°C. where heat is being lost very rapidly, a formula of 50% R + 50% S may give better estimates. This means, however, that very careful measurements of average skin temperature must be made at these lower temperatures if good estimates of change in average body temperature are to be made.

9. Radiation, convection, vaporization. The partition of the total heat loss at different temperatures is shown in figure 4. Vaporization loss at 22°C. amounts to only 15% of the total

and it is probable that more than half of this is from the lungs. At 32°C. just before the onset of sweating the vaporization loss was 40% of the total. This increase in importance of vaporization is the result of decreasing heat loss rather than increase in actual amount of vaporization. Radiation at 22°C. is greater in magnitude than the heat production and amounts to more than 70% of the total heat loss. Between 35°C. and
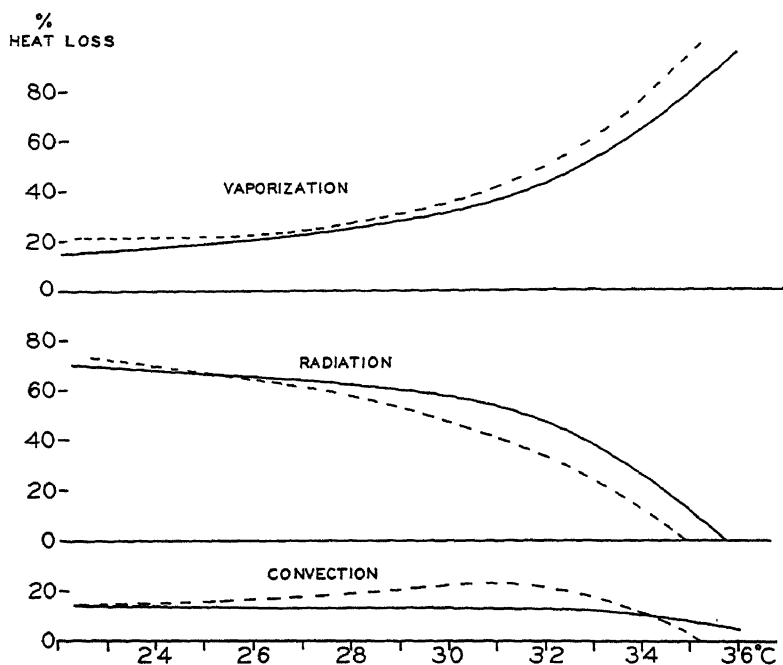
Fig. 4   Average partitions of heat loss at different calorimeter temperatures of men and women. Abscissae, calorimeter temperature; ordinates, per cent of total heat loss. Solid line, women; dashed line, men.

36°C. radiation becomes zero, this being the point at which the average skin temperature is the same as the calorimeter temperature. The radiation loss in per cent is maximal under the conditions of these experiments, the subjects lying nude and motionless. With activity the actual amount of radiation will increase in the cold atmospheres and decrease in the warm atmospheres. The per cent of heat lost by convection increases

slightly with lower calorimeter temperatures but ranges between 5% and 15% throughout the experimental range. Convection loss falsely indicates a positive value even when the skin temperature is below that of the calorimeter. This artifact is probably due to the technical difficulty of operating the calorimeter at such high temperatures. Values of heat loss measured in this range may be in error by 5 or 6 calories per hour, an amount sufficient to account for the anomalous results shown in the chart.

10. Comparison of the responses of nude and motionless male and female subjects to different calorimeter temperatures. In one respect there was no demonstrable sex difference in the responses to environmental temperature, namely, the rate at which the body cools per degree difference in skin and calorimeter temperature. Aside from this factor, however, there were significant differences in all of the quantities studied. Figure 5 shows the comparison of average values.

The rectal temperature of the men decreased very uniformly with environmental temperature, whereas that of the women showed a low point at about 31°C. On either side of this environmental temperature the rectal temperature of the women was higher and in some of the cool experiments the women had distinctly higher internal temperatures.

In the environmental temperature range below 30°C. the average surface temperature of the women changed 0.57°C. for a change of 1 degree in calorimeter temperature. The average surface temperature of the men changed 0.48°C. on the same basis. Thus, in the cool zone the surface temperature and the average body temperature of the women were lower than that of the men exposed under the same conditions. In the warmer atmospheres the surface temperature of the women rose to a higher level than that of the men (1 to 1½ degrees). Thus, their average body temperature was warmer under these conditions than that of the men. This difference in the change of surface temperature with environmental temperature accounts for the differences in per cent heat lost by radiation, shown in figure 4, in the cold and warm atmospheres. This

difference is much more pronounced at the higher temperatures in which the women radiate much more of their heat than do the men.
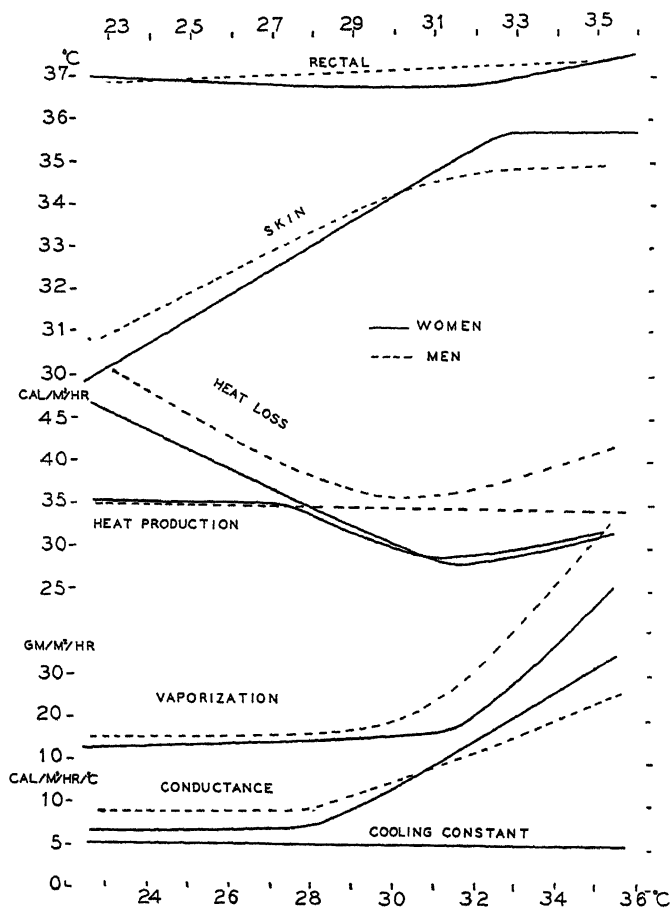
Fig. 5   Comparison of average values of various factors for women and men at different calorimeter temperatures.

The heat loss of the women is on an average 3 to 4 cal./hr./M² less than that of the men in the temperature range below 28°C. The rate of increase of heat loss as the calorimeter temperature is lowered is approximately the same for both sexes, that is, the heat loss increases 2.2 cal./hr./sq.M for each degree

fall in calorimeter temperature for the women and 2.4 cal./ degree for the men. The striking difference in the heat loss curves is seen in the low values reached by the women. Values as low as 25 cal./hr./M^2 have been found for the women, whereas 36 cal. is the low point for men. The low point of heat loss for the men was found at calorimeter temperature 30°C. and for the women at 31.5°C.

The basal heat production of the women and the men was the same and level at 35 cal./hr./M^2 for calorimeter temperatures lower than 27°C. In both sexes no change in metabolism was observed in the colder range until the actual onset of a chill, and no "chemical regulation" as a response to low temperature was observed in this range. Above 28°C. the heat loss for women was the same as the heat production and both decreased with higher temperature to the low point at 30°C. to 32°C. No such phenomenon was observed for the men whose basal heat production was constant throughout the experimental range. That the change in the basal heat production which was observed in women was a real response to environmental temperature is supported by the corresponding changes observed in the heat loss for the women and not for the men. The range of vasomotor regulation of the women is seen to be larger than that of the men as the latter have only the 2-degree range from 29°C. to 31°C., whereas the women appear to have the 5-degree range from 27°C. to 32°C.

Vaporization for the men was higher in the cold zone than for the women. This may be due to the fact that women can more completely cut off the peripheral circulation and a dryer skin surface is a consequence of slower transudation in women. The men began to sweat visibly at about 30°C. whereas the women, because of their lowered heat production, did not need to sweat until the temperature was between 32°C. and 33°C. The amount of sweating in the women was progressively less than in the men, as the temperature was raised. The per cent heat lost by sweating for the women was also less than for the men at all temperatures except between 27°C. and 29°C. It has been pointed out that a definite relationship might be

expected between the basal heat production in men and the water vaporized as long as the temperature did not rise above 29°C. For the women the temperature would have to be lower than 27°C. and even there the relationship would be unsatisfactory as the water loss decreased with lower temperature.

The thermal conductance of the superficial tissue would be expected to be higher for the men than the women from a consideration of the levels of heat loss for the sexes. The conductance for the women was estimated as 2.2 cal./hr./M²/°C. lower than for the men, at 23°C. On the basis of the measured specific thermal conductivity of fat and muscle tissue, this corresponds to about 5 mm. of extra superficial tissue for the women. The conductances of both sexes increased slightly as the calorimeter temperature was raised. Between 28°C. and 29°C. both sexes showed definite vasomotor response in the superficial vessels. Three of the women subjects had the same degree of response as the men. Three of the women, however, increased their blood flow at twice that rate. Both the level of conductance and the rate of increase may represent individual responses to temperature in these warm atmospheres. Racial differences, acclimatization, and other factors should be important. So far as conductance is concerned, the only real difference between the sexes was found in the lower temperature range.

SUMMARY

Eight normal women have been studied in the calorimeter of the Russell Sage Institute of Pathology in regard to the mechanism of heat loss and heat production at environmental temperatures ranging from 22° to 36°C. The women were lying nude under basal conditions on a comfortable bed within the calorimeter. The results have been compared with those obtained on two men, using exactly similar procedure. The basal metabolic rate of the women in the colder zone was about 35 cal./sq.m./hr. and approximately the same as that of the men. In the warmer zone the women showed a marked drop in basal metabolism until it averaged 30.9 cal./sq.m./hr. at

31°C. The men showed no such drop. This indicates a type of regulation of body temperature which was not found in the men whose metabolism had remained level. In the women the fall in basal metabolism was concomitant with marked decrease in heat loss in the comfort zone, reaching at 31.5°C. as low a level as 28.5 cal./sq.m./hr. The lowest point observed for the men was 34.9 cal./sq.m./hr. In the warmer zones there was considerable variation in the individual response.

The average skin temperature for the women in the hot zone was higher than that for the men by 1.5°C. and lower in the cold zone by 1.0°C. The conductance of the peripheral tissues for the women was 20% lower than that of the men in the cold experiments, representing 20% more insulation against cold. Sweating was less marked in the women and did not begin until the environmental temperatures were higher than was the case for the men. In general, there were slight differences in every single factor of temperature regulation, and in all respects the women had physiological advantage, but especially in the reduction in heat loss and heat production in the warmer comfort zone.

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A QUANTITATIVE STUDY OF VITAMINS IN THE RUMEN CONTENT OF SHEEP AND COWS FED VITAMIN-LOW DIETS

IV. PANTOTHENIC ACID

L. W. McELROY AND HAROLD GOSS

Division of Animal Husbandry, College of Agriculture,
University of California, Davis

In previous communications (McElroy and Goss, '40 a, '40 b, '40 c; McElroy and Jukes, '40) we have reported that riboflavin, vitamin K, pyridoxine, thiamine and the anti egg-white-injury factor were found in the rumen contents of animals fed a ration deficient in these vitamins. In the present investigation evidence was found for the formation of pantothenic acid in the rumen of the experimental sheep and cows.

EXPERIMENTAL

The care and treatment of the experimental ruminants has been described in an earlier paper (McElroy and Goss, '40 a).

Pantothenic acid assays. The "filtrate factor" method of Jukes ('37) was used in estimating pantothenic acid. Chicks were placed on an ordinary mixed diet for 6 to 8 days after hatching and then depleted for 8 to 10 days on heat-treated diet 80G composed of natural foodstuffs. The chicks were then weighed and divided into groups. Each series was controlled by a group on the basal diet made up of 100 gm. of the heated diet 80G + 4 cc. of a rice bran filtrate which had previously been shown to produce a maximal growth response when fed at a level of 3.5 cc. per 100 gm. of diet. When maximal growth response was produced under the conditions of the assay the diet was said to contain at least 1 filtrate factor unit per gram.

Jukes ('39) has shown that 1 filtrate factor unit is equivalent to 14 µg. of pantothenic acid.

The results of an assay of dried sheep rumen and reticulum contents and of the ruminant ration are summarized in table 1. The reader is referred to the original paper of Jukes ('37) for details of the method of calculating results. The pantothenic acid activity of the rumen and reticulum contents was estimated to be 5 chick units (70 µg.) per gram as compared with less than 0.2 chick units (2.8 µg.) per gram for the ruminant ration.

TABLE 1

Pantothenic acid assay of ruminant ration and of dried sheep rumen and reticulum contents. Supplements were added to diet 80G. Ten chicks were included in each group

SUPPLEMENT IN 100 GM. OF DIET	AVERAGE GAIN IN WEIGHT IN 8 DAYS	NUMBER OF CASES OF SPECIFIC DERMATITIS AFTER 8 DAYS
	gm.	
1. None	1.7	9
2. 15 gm. ruminant ration	— 6.0	5
3. 15 gm. rumen and reticulum contents	29.2	0
4. 4 cc. rice bran filtrate (+ control)	38.4	1 (mild)

SAMPLE OF CALCULATIONS:

$$\text{Pantothenic acid per gram} = \frac{29.2 - 1.7}{(38.4 - 1.7)\,0.15} = 5 \text{ chick units or } 70 \text{ } \mu g.$$

The same deficient ration which was fed to sheep was also fed to cows. One of the experimental animals, no. 557, had a fistula into the rumen as described in an earlier paper (McElroy and Goss, '40 a). Samples of rumen contents from two of these animals and milk from one of them were assayed for pantothenic acid. Typical results are summarized in tables 2 to 4. From table 2 it was estimated that the sample of rumen contents obtained from the non-fistulated cow no. 706 contained 61 µg. of pantothenic acid per gram dry matter. This sample contained a large amount of sand and grit (McElroy and Goss, '40 c) and consequently it is believed that this is an excessively low estimate of the true value during the experimental period. More representative samples of rumen contents were obtained from the fistulated cow no. 557 and a

typical assay of a composite sample of this material is given in table 3. The pantothenic acid content of this sample was estimated to be 94 µg. per gram.

Table 4 shows that dried skim milk prepared from the milk of cow no. 557 contained 46 µg. of pantothenic acid per gram.

TABLE 2

Pantothenic acid assay of dried rumen contents from non-fistulated cow no. 706. Supplements added to heated diet 80G

SUPPLEMENT IN 100 GM. OF DIET	NUMBER OF BIRDS	AVERAGE GAIN IN WEIGHT IN 11 DAYS	NUMBER OF CASES OF SPECIFIC DERMATITIS AFTER 11 DAYS
		gm.	
1. None	3	—1.3	3
2. 12 gm. dried rumen contents	6	32.4	2
3. 4 cc. rice bran filtrate	8	62.7	2 (mild)
Estimated pantothenic acid, 61 µg. per gram.			

TABLE 3

Pantothenic acid assay of dried rumen contents of fistulated cow no. 557. Supplements added to heated diet 80G.

SUPPLEMENT IN 100 GM. OF DIET	NUMBER OF BIRDS	AVERAGE GAIN IN WEIGHT IN 10 DAYS	NUMBER OF CASES OF SPECIFIC DERMATITIS AFTER 10 DAYS
		gm.	.
1. None	5	5.8	5
2. 8 gm. dried rumen content	7	34.0	2
3. 6 cc. rice bran filtrate	7	58.0	0
Estimated pantothenic acid, 94 µg. per gram.			

TABLE 4

Pantothenic acid assay of dried milk, fistula cow no. 557. Supplements added to heated diet 80G.

SUPPLEMENT IN 100 GM. OF DIET	NUMBER OF BIRDS	AVERAGE GAIN IN WEIGHT IN 12 DAYS	NUMBER OF CASES OF SPECIFIC DERMATITIS AFTER 12 DAYS
		gm.	
1. None	6	10.9	6
2. 20 gm. dried skim milk [1]	7	40.8	0
3. 4 cc. rice bran filtrate	7	55.7	0
Estimated pantothenic acid, 46 µg. per gram.			

[1] The skim milk used was prepared from milk collected between the seventeenth and twenty-fourth and between the forty-fifth and forty-ninth days of lactation. Equal weights of the two preparations were combined to make the 20% supplement.

During the period when this milk was being collected this cow was giving 20 pounds of milk per day. Assuming that about 10 pounds of milk are required to produce 1 pound of dry skim milk, then the daily excretion of pantothenic acid by way of the milk would be approximately 41 mg. The maximum food consumption of the cow during this period was 6 kg. per day of a ration containing less than 2.8 mg. of pantothenic acid per kilogram, or a total of about 17 mg. per day as compared with a daily excretion in the milk of 41 mg.

In an earlier paper (McElroy and Goss, '40 b) we discussed the question of differential passage of materials from the rumen as a possible cause of the increase in the vitamin potency of rumen contents. It does not seem to appear possible that a twenty- to thirtyfold increase in pantothenic acid concentration of the rumen contents over that of the ration fed could be explained in this manner. Our results with milk and with rumen contents indicate that appreciable quantities of pantothenic acid may be formed in the rumen, probably by bacterial growth.

SUMMARY

1. Sheep and cows were fed a vitamin B complex low diet containing less than 2.8 µg. of pantothenic acid per gram. The rumen and reticulum contents of the sheep were found to contain 70 µg. of pantothenic acid per gram dry substance or a twenty-fivefold increase over the ration fed.

2. Samples of rumen contents from two cows fed the deficient ration contained from twenty to thirty times as much pantothenic acid as did the ration fed.

3. The amount of pantothenic acid excreted by way of the milk from one of the cows was estimated to be twice as great as could be accounted for in the ration fed.

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URINARY EXCRETION OF THIAMINE ON HIGH FAT AND HIGH CARBOHYDRATE DIETS [1,2]

WILLIAM M. CAHILL

Department of Physiological Chemistry, Wayne University College of Medicine, Detroit, Michigan

ONE FIGURE

As early as 1914 Funk made the observation that pigeons on a diet deficient in thiamine and high in carbohydrate developed polyneuritis earlier than pigeons on a diet high in fat but similar in other respects. Since that time a number of research workers have investigated further the relationship between the thiamine requirement of animals and the percentage of carbohydrate in the diet. Evans and Lepkovsky ('29), for instance, reported that young rats required less thiamine for growth on thiamine-low diets when some of the carbohydrate of the diet was replaced by fat. In 1937 Salmon and Goodman reexamined the question and their results confirmed earlier reports on the "thiamine-sparing" action of fat. More recently Stirn, Arnold and Elvehjem ('39) after a careful experimental study of the problem report that when carbohydrate in a thiamine-deficient ration is isocalorically replaced by fat the resultant diet will ameliorate the condition of polyneuritic rats. It is interesting to note that experiments

with dogs indicate that these animals also require more thiamine on a high carbohydrate than on a high fat regimen (Arnold and Elvehjem, '39).

The purpose of the present investigation was to determine whether or not alterations in the ratio of fat to carbohydrate in the diet of human adults are reflected in the urinary thiamine output when the dietary intake of thiamine remains constant. The urinary thiamine excretion has been found to be a useful index of thiamine deficiency (compare Robinson, Melnick and Field, '40), and it is important that all factors which might possibly influence the thiamine excretion be studied. The excretion of thiamine in the urine is a sensitive index of the vitamin intake, a change from a diet adequate in thiamine to one inadequate in this vitamin being promptly reflected by a decrease in the urinary output of vitamin B_1 (compare Melnick, Field and Robinson, '39). It was, therefore, thought possible that if a high carbohydrate diet resulted in an increased demand for thiamine by the body this fact might be indicated by a change in the urinary output of thiamine within a relatively short period.

There are conflicting reports in the literature regarding the effect of a high carbohydrate diet on the urinary thiamine excretion. Schroeder ('39) reported that large doses of sugar given daily to two patients on a normal diet eventually caused the urinary thiamine output to become nil. On the other hand, in a report which appeared after the present investigation was begun Wang and Yudkin ('40) conclude that changes in the carbohydrate content of the diet affect the urinary thiamine output very little, if at all.

EXPERIMENTAL

In the present investigation the urinary thiamine excretion of individuals on comparable high carbohydrate and high fat diets was studied. The subjects were all males from 20 to 30 years of age. In one series of experiments, on each of 3 consecutive days individuals were given a loaf, or a definite fraction of a loaf, of bread and 1000 calories of sucrose.

In addition, 4 gm. of brewers' yeast were eaten by the subjects daily. On the third day a 24-hour sample of urine was collected for determination of its thiamine content. During a similar 3-day period, the same individuals were given a diet identical in all respects except that the sucrose was replaced iso-calorically with butter. The amount of bread eaten each day by any one individual was exactly the same during the two 3-day periods. On the third day the urine was collected as before and the thiamine in the sample was determined chemically essentially according to the method of Melnick and Field ('39). The 24-hour urine samples of any one individual—i.e., the urine collected during a period when the diet was high in fat and when it was high in carbohydrate—were analyzed for thiamine on the same day to make conditions as uniform as possible. Melnick and Field ('39) have shown that when the urine is preserved with acid and toluol no loss of thiamine occurs for a considerable period of time.

The results of the experiment are shown in the first part of table 1. Although the urinary thiamine output varies from individual to individual, it was found that an increase in fat at the expense of carbohydrate in the diet of any one individual did not significantly alter the urinary thiamine excretion. Changes in the ratio of the calories of fat to the calories of carbohydrate in the diet varying as widely as 1:0.75 to 1:50 did not significantly influence the urinary excretion of thiamine in these experiments.

In a comparable study one individual (W.M.C.) was given an experimental diet for 12 days. For alternate 3-day periods the diet was first high in carbohydrate and then in fat. The diet consisted of "whole" wheat bread, 4 gm. of dried yeast, and either 1000 calories of fat or of carbohydrate, depending upon the period. Here again radical changes in the ratio of fat to carbohydrate in the diet did not change significantly the output of thiamine in the urine. The only noteworthy alteration was a moderate increase in the excretion of thiamine as the experiment progressed.

It was thought that perhaps in the foregoing experiments the high carbohydrate diets did not depress the thiamine output because the vitamin B_1 content of the diets was too high. In a second series of experiments, therefore, individuals were given thiamine-low diets consisting of white bread and butter,

TABLE 1

Urinary excretion of thiamine by normal adults on comparable high fat and high carbohydrate diets

SUBJECT	WEIGHT	DIETARY THIAMINE INTAKE	DAILY DIET					RATIO OF FAT TO CARBOHYDRATE IN DIET	THIAMINE EXCRETED ON 3RD DAY
			Yeast [1]	Bread [2]	Butter	Sucrose	Total		
	kg	µg./24 hrs.	Cal	Cal.	Cal.	Cal.	Cal.	Cal.F:Cal.C	µg./24 hrs.
C.A.K.	78.5	1420	14	1303	1000	....	2317	1: 1.08	284
		1420	14	1303	....	1000	2317	1:43.2	304
E.A.H.	67	1480	14	1418	1000	....	2432	1: 1.09	229
		1480	14	1418	....	1000	2432	1:41.6	222
H.B.	68.5	1290	14	1073	1000	....	2087	1: 0.84	256
		1290	14	1073	....	1000	2087	1:47.7	264
M.F.P.	63	1480	14	1418	1000	....	2432	1: 1.09	193
		1480	14	1418	....	1000	2432	1:41.6	174
W.W.	79	1210	14	957	1000	....	1971	1: 0.75	156
		1210	14	957	....	1000	1971	1:50.8	168
W.M.C.	68	1420	14	1303	1000	....	2317	1: 1.08	270
		1420	14	1303	....	1000	2317	1:43.2	298
E.R.J.	65.5	155	..	1371 [3]	1000	....	2371	1: 1.04	87
		155	..	1371	....	1000	2371	1:34.6	89
H.W.	70	170	..	1492 [3]	1000	....	2492	1: 1.13	78
		170	..	1492	....	1000	2492	1:33.2	71
R.L.	69	170	..	1492 [3]	1000	....	2492	1: 1.13	71
		170	..	1492	....	1000	2492	1:33.2	78
L.K.L.	72	170	..	1492 [3]	1000	....	2492	1: 1.13	66
		170	..	1492	....	1000	2492	1:33.2	66

[1] Brewers' yeast (4 gm.). This yeast contained 168 µg of thiamine per gram. We are indebted to Dr. Daniel Melnick of the University of Michigan for determining the total thiamine content of the yeast.

[2] Unless otherwise noted all the bread used in this study was a "whole" wheat bread obtained from a small bakery. The flour used in its preparation consisted of three-quarters whole wheat and one-quarter white flour. The fat and carbohydrate content of the bread was calculated from tables compiled by Sherman ('38) on the basis that the bread was three-quarters whole wheat and one-quarter white. The thiamine content of the bread was also calculated on this basis from tables in Hawk and Bergeim ('37).

[3] White bread.

or of white bread and sugar. Yeast was omitted from these diets. The experimental procedure was the same as in the first series of experiments. The results of this study are also shown in table 1.

The thiamine intake of the second group of individuals studied was much less than that of the first group. This fact is well reflected in the much lower output of thiamine in the urine of those individuals receiving an inadequate amount of thiamine in the diet. Here again, however, it was found that radical changes in the ratio of fat to carbohydrate in the diet were not reflected by any significant variation in the urinary thiamine output.

In a study bearing on the diagnosis of thiamine deficiency Robinson, Melnick and Field ('40) have shown that the percentage of an oral dose of 5000 μg. of thiamine which is excreted in the urine is lower in the case of vitamin B_1-deficient individuals than in normal subjects. Thiamine-deficient individuals excrete less than 7% of the test dose. Advantage was taken of this test in another experiment designed to study the effect of carbohydrate on the urinary thiamine output. An individual (W.M.C.) was given a thiamine-low diet consisting of white bread and 1000 calories of butter daily. The urinary thiamine was determined on alternate days. On the ninth day of the experiment 5000 μg. of thiamine were taken orally by the subject and the urinary thiamine output of the following 24-hour period was similarly determined. After an interval of 2 weeks, during which time the subject consumed a normal diet, the experiment was repeated with the fat in the diet replaced isocalorically by sucrose. The amount of bread eaten daily during the two periods was the same. The results of the study are shown in figure 1.

This latter study yielded results comparable to those of the other experiments reported in this paper. Whether the low thiamine diet was high in fat or high in carbohydrate, the picture of urinary thiamine excretion was essentially the same. In both cases the excretion of thiamine dropped rapidly and reached a similar level. In the case of the experiment with

a high fat diet, 5.9% of the oral dose was excreted in the urine, while in the case of the study with a high carbohydrate diet 5.6% of the oral dose was excreted.
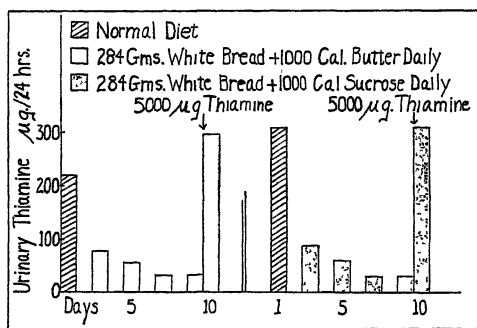
Fig. 1 Urinary excretion of thiamine administered orally after a period of depletion on comparable high fat and high carbohydrate diets.

DISCUSSION

The choice of the rather simple experimental diets was dictated by the desire to have as few variables as possible, as well as by the necessity of having easily reproducible diets of known composition which could be eaten by the cooperating subjects with a minimum of effort on their part, and which would also entail minimum interference with their time schedule. While the diets were not altogether adequate in all the known essential food factors, the rather short experimental period would seem to excuse this. On the other hand, it was thought that if any significant alterations of the urinary thiamine excretion were effected by great distortions of the ratio of fat to carbohydrate in the diet they would make themselves apparent within the experimental period. This seems probable in view of the fact that alterations in the amount of thiamine ingested reflect themselves almost immediately by changes in the urinary thiamine output as clearly shown in figure 1.

It has been said that "fat spares thiamine," and while it seems established that under certain conditions the thiamine requirement of growing animals is influenced by the percent-

age of fat in the diet, it is interesting to note that analyses of the tissues of rats, chickens, and pigs fed on rations deficient in thiamine and high and low in fat, respectively, have indicated that the thiamine content of the tissues is dependent upon the thiamine content of the ration and not upon the fat content (Kemmerer and Steenbock, '33). The amount of fat or carbohydrate in diets of like thiamine content did not differentially have any influence on the thiamine content of the tissues. The observations reported in the present study are somewhat comparable. The finding in the present investigation that, under the conditions described, alterations of the fat-carbohydrate ratio of the diet have no significant effect upon the urinary elimination of thiamine of adult human subjects does not, of course, gainsay the conceivable significance of this ratio in nutrition, particularly over long periods and with growth as part of the picture.

The present results indicate that in tests involving a study of the urinary thiamine excretion of patients, no concern need be given to the fat-carbohydrate ratio of the diet eaten just prior to the determination. This fact is of practical importance, since tests of this nature are used as aids in the diagnosis of thiamine deficiency (compare Robinson, Melnick and Field, '40).

SUMMARY

In an attempt to determine whether the carbohydrate content of the diet, like the thiamine content, influences the urinary output of thiamine, a study was made of the urinary thiamine excretion of adult individuals on comparable high fat and high carbohydrate diets. In all of the experiments reported it was found that even great alterations in the ratio of fat to carbohydrate in diets of the same thiamine content did not significantly affect the urinary thiamine excretion. This was true whether the thiamine intake was adequate or fairly low. A study of the excretion of thiamine administered orally after a period of depletion on comparable high fat and high carbohydrate diets also revealed no differential

influence of fat or carbohydrate on the urinary thiamine output. The bearing of these findings on the validity of tests for thiamine deficiency which are based on a determination of the amount of thiamine excreted in the urine is pointed out.

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THE OPTIMUM LEVEL OF PROTEIN INTAKE FOR THE GROWTH AND FATTENING OF SWINE [1]

R. C. MILLER AND T. B. KEITH

Department of Agricultural and Biological Chemistry and Department of Animal Husbandry, Pennsylvania State College, State College

INTRODUCTION

The choice of the level of protein when expressed as a percentage of the ration to be fed to pigs, presents a major problem in the nutrition of this species. Contributing factors to the complexity of this problem are: (a) the relatively rapid growth rate of swine, which exceeds that of any other class of livestock, (b) possible decreasing requirements for protein during the progress of growth, and (c) economic considerations, especially those concerning the relatively high price of protein supplements.

Carroll and Mitchell and associates ('32, '34, '35, '36, '37) have investigated by means of growth and nitrogen balance studies the question of the changing requirements of pigs at different stages of growth, and have shown that for pigs weighing less than 100 pounds the ration should contain more than 17% of a protein mixture obtained from corn, tankage, soybean oil meal or linseed oil meal, and alfalfa meal. For pigs weighing 150 pounds, 17% of protein was found to be adequate and 15% was enough for pigs weighing 175 to 200 pounds. For pigs weighing 40 to 100 pounds, protein levels

up to and exceeding 22% were indicated, by nitrogen balance studies, as being necessary for maximum growth.

Recently Carroll and Burroughs ('39) in a preliminary report of dry-lot group-feeding trials have shown that young pigs must receive a higher percentage of protein than is sufficient for heavier pigs, and their data suggest that the ration of 50-pound pigs should contain at least 18 to 20% of protein or perhaps more. Between the weights of 75 and 100 pounds their data do not indicate any advantage in gains for more than 15% of protein in the ration, and 12% of protein is indicated for pigs weighing from 150 to 200 pounds.

Woodman, Evans, Callow and Wishart ('36) have concluded from the results of individual feeding trials and group feeding tests that a feeding treatment providing 12% of fish meal (17.48% crude protein) from weaning to 90 pounds live weight, 10% of fish meal (15.93% crude protein) from 90 to 150 pounds, and 5% of a mixture of equal parts of meat meal and ex. soya-bean meal (12.25% crude protein) in the final period will supply all of the digestible protein for the maximum rate of growth permitted by the net energy content of the ration. Higher amounts of protein in the ration were of no benefit and actually depressed the rate of live weight increase in some cases.

From these reports it would appear that, while the minimum protein levels which will produce good growth in pigs are fairly well recognized, the relative value of higher levels of protein intake is not well established. Experiments dealing with the general problem of how much protein it is nutritionally advisable to feed to growing pigs have been conducted by the authors since 1936 and this report presents some of the results of this research.

EXPERIMENTAL

Four experiments were conducted. The number of pigs used in each experiment, the protein levels compared, and the approximate intervals of body gain studied are given in table 2.

The composition of the rations is given in table 1. Number 2 yellow corn was used throughout the experiments. The tankage was a 36% protein, steam rendered by-product of a local abattoir and was relatively high in bone content. The soybean oil meal used in these experiments was processed by the expeller method from beans of the Illini, Manchu, Dunfield, Richland and Mandell varieties. The alfalfa meal was produced from local sun-dried hay by passing it through a hammer

TABLE 1

Composition of rations

INGREDIENT	EXPERIMENT 1		EXPERIMENT 2		
	PER CENT PROTEIN		PER CENT PROTEIN		
	10	15	15	20	25
	%	%	%	%	%
Yellow corn	93	76	76.5	57.5	42.5
Tankage	1	15	15	20	18
Soybean oil meal	1	4	4	18	35
Alfalfa meal	4	4	4	4	4
Salt	0.5	0.5	0.5	0.5	0.5
Ground limestone	0.5	0.5	....	....	....

INGREDIENT	EXPERIMENT 3			EXPERIMENT 4		
	PER CENT PROTEIN			PER CENT PROTEIN		
	12	17	22	17	22	27
	%	%	%	%	%	%
Yellow corn	87.5	69.5	51.5	65.5	45.5	36.5
Tankage	4	13	22	12	22	33
Soybean oil meal	4	13	22	12	22	20
Alfalfa meal	4	4	4	10	10	10
Salt	0.5	0.5	0.5	0.5	0.5	0.5

mill. One-half of 1% of salt and 1% of ground limestone were included in the rations of experiment 1. The limestone was omitted in experiments 2, 3 and 4, since the chemical analysis indicated that the additional calcium was not necessary and did not influence the calcium-phosphorus ratio to any marked extent. In addition direct sunlight was available in all of the experiments.

The rations were designed to provide the different levels of protein intake indicated in table 1. No determinations were made of the digestibility of the protein or of its biological

value. Rather the scope of these experiments was limited to
a study of the relative values of the gross levels of protein
intake for pigs of different ages, it being felt that a study of
the influence of the level of protein intake and of the stage
of development of pigs, on the digestibility and biological
value of the rations constituted a separate problem for later
study. However, due to the similarity in the constitution of
the various rations it is not thought that their digestibilities
are markedly different.

Pigs of the Poland China, Chester White, Duroc Jersey
and Berkshire breeds were used. They were housed in an
experimental feeding house provided with a concrete floor and
having outside concrete runways. They were fed as matched
pairs in experiment 1 and as matched triplets in the other
trials. The experimental pairs or triplets were selected to be
of the same breed, sex, age and of similar weight, and members
of each pair or triplet were fed the same daily quantity of
feed. The amount of feed allotted daily to each member of a
pair or triplet was determined by the feed consumption of
the pig in each pair or triplet which consumed the smallest
quantity of feed; hence, the feed intake was probably re-
stricted to some extent for one member of each pair and two
members of each triplet. The pigs were fed three times daily
in individual stalls especially constructed for these experi-
ments. They were weighed weekly, and the initial and final
weights as given in table 2 are the averages of weights taken
on 3 successive days.

RESULTS AND DISCUSSION

Table 2 is a summary of the results. An analysis by variance
was made on the data for individuals for the average daily
gains and for the feed requirement for 100 pounds of body
gain. There are also given the differences between means
which are required to show significance at the 5% point of
probability.

In experiment 1 the gains for all of the pigs on the 15%
level of protein exceeded those of the pigs on the 10% level
and were attained with less feed per unit of gain.

The data for experiment 2 covering the growth span from 84 to 200 pounds, also have been broken down to cover other intermediate weight increments. Gains and food efficiency were less for pigs on the 25% protein level in a number of instances. The comparison for the weight interval 127 to 200 pounds was made with only seven pigs at each level of protein intake, there being only seven triplicates which attained the required initial weight of 127 pounds in comparable time intervals. Significant differences were not obtained with respect to gains or feed requirement between the 15 and 20% protein levels, both of which are indicated as better than the 25% level.

Lighter pigs weighing 40 pounds were used in experiment 3. The 12% protein level proved unsatisfactory for these pigs. On this level three pigs failed to gain in a manner satisfactory enough to be included in the comparison, and the feed requirement for the remaining nine pigs was relatively high as was the variation between individuals. Consistent results were obtained favoring the 17% protein level, although on the 22% protein level similar results were obtained in some of the comparisons. The failure of individual pigs to attain the required weights in time intervals to give valid comparisons necessitated making comparisons, in some cases, between unequal numbers of individuals on the respective protein levels. The number of individuals used on any one level is, therefore, some indication of the merit of that particular protein level.

A summary of the results shows that neither an increased rate of body gain nor increased feed efficiency results from a 25% protein level of feeding as compared with either a 15 or 20% level. A 10% protein level was shown to be inferior to a 15% level as judged by body gains and by feed efficiency. Results obtained with 12 and 22% protein levels showed no significant differences favoring either level with respect to growth. Both of these levels, however, seemed to be less satisfactory than the 17% protein level judging by both the average daily gains of the pigs and the efficiency of their food utilization.

TABLE 2

Summary of average weights, body gains and feed requirements of pigs fed rations of different protein content

NUMBER OF PIGS	AMOUNT OF PROTEIN IN RATION	AVERAGE AGE AT BEGINNING	LENGTH OF EXPERIMENTAL PERIOD	INITIAL BODY WEIGHT	MEAN AVERAGE DAILY GAIN	MEAN AVERAGE DAILY RATION	FEED FOR EACH 100 LBS. GAIN
	%	days	days	lbs.	lbs.	lbs.	lbs.
Experiment 1 — 130 to 180 lbs.							
10	10	222	39	131	1.08	4.66	447
10	15	193	39	129	1.43	4.65	326
			standard error		.10		17
		difference required to be significant			.31		58
Experiment 2 — 84 to 127 lbs.							
13	15	136	35	82	1.34	3.53	269
13	20	121	31	85	1.39	3.42	252
13	25	125	33	86	1.28	3.58	284
			standard error		.05		15
		difference required to be significant			.16		45
		84 to 156 lbs.					
13	15	136	53	82	1.39	3.88	281
13	20	121	50	85	1.47	3.83	265
13	25	125	54	86	1.31	3.89	300
			standard error		.05		16
		difference required to be significant			.13		48
		127 to 200 lbs.					
7	15	166	39	128	1.76	4.71	268
7	20	154	46	128	1.73	4.58	265
7	25	162	45	127	1.49	4.60	316
			standard error		.06		19
		difference required to be significant			.19		59
		84 to 200 lbs.					
13	15	136	77	82	1.55	4.28	276
13	20	121	77	85	1.55	4.28	276
13	25	125	77	86	1.38	4.29	314
			standard error		.04		12
		difference required to be significant			.12		33
Experiment 3 — 40 to 74 lbs.							
9	12	77	76	40	.45	1.56	375
12	17	70	72	38	.50	1.43	301
12	22	73	70	41	.51	1.31	289
			standard error		.03		29
		difference required to be significant			.10		85
		74 to 156 lbs.					
7	12	156	73	73	1.14	3.25	285
12	17	144	58	75	1.43	3.28	229
11	22	145	69	75	1.18	3.29	274
			standard error		.04		11
		difference required to be significant			.15		33

TABLE 2—*continued*

NUMBER OF PIGS	AMOUNT OF PRO-TEIN IN RATION	AVERAGE AGE AT BEGIN-NING	LENGTH OF EXPERI-MENTAL PERIOD	INITIAL BODY WEIGHT	MEAN AVERAGE DAILY GAIN	MEAN AVERAGE DAILY RATION	FEED FOR EACH 100 LBS. GAIN
	%	days	days	lbs.	lbs.	lbs.	lbs.
		156 to 213 lbs.					
4	12	213	35	155	1.68	4.64	283
11	17	202	33	156	1.83	4.61	257
6	22	214	38	155	1.52	4.66	309
				standard error	.06		13
		difference required to be significant			.17		38
		130 to 210 lbs.					
5	12	209	53	130	1.59	4.63	282
12	17	187	49	131	1.84	4.52	245
10	22	193	55	128	1.56	4.47	302
				standard error	.07		11
		difference required to be significant			.20		32
		40 to 210 lbs.					
9	12	77	166	40	.88	2.79	332
12	17	70	166	38	1.09	2.76	252
11	22	67	166	41	.98	2.75	282
				standard error	.04		16
		difference required to be significant			.13		48
Experiment 4 — 33 to 70 lbs.							
10	17	78	54	33	.75	1.85	271
10	22	78	51	32	.74	1.94	270
10	27	75	49	34	.78	1.98	254
				standard error	.12		10
		difference required to be significant			.37		31

From the results obtained with pigs of lighter weight, 80 to 125 pounds, 40 to 74 pounds and 33 to 70 pounds, no single level of protein intake could be designated as being the most satisfactory for body gain but the 12% level was definitely unsatisfactory with respect to feed requirement. Woodman and associates ('36) found that "extra protein" was of no value in improving the rate of growth in the period from weaning to 90 pounds, a period in which the value of such protein might be expected to be manifested. It is possible that other factors, some of which may be unrecognized, may influence the growth of pigs at this stage of their growth, more than it is influenced by the protein level, providing that the protein is adequate from the viewpoint of quality. The results of these experiments do not indicate any deficiency in the

quality of the protein or any of the rations with the possible exception of the 10% protein ration used in experiment 1.

The results obtained with the lighter pigs are not surprising in view of the difficulties often encountered with pigs of this weight. The general nutritional requirements of such pigs are not very well established and for this reason many workers select pigs for growth studies when they weigh 70 to 80 pounds rather than at weaning time. In the interim between weaning and being placed on experiment, these larger pigs will have had access to feeds which do not impose on them the restrictions of an experimental diet and thus may be able to build up reserves of dietary essentials which may have been depleted during the suckling period. The less thrifty individuals are eliminated during this period and the surviving pigs which attain a weight of 70 to 80 pounds and which are then placed on the experimental regime, can usually be depended on to give a more satisfactory growth response. The findings of Chick and associates ('38 a, b, c) and Hughes ('38, '39) with respect to the requirements of pigs for members of the vitamin B complex illustrate the paucity of knowledge relative to the requirements of swine for specific dietary factors.

Woodman and associates ('36) have conducted individual feeding tests in a manner similar to that of these experiments. In addition these investigators conducted group feeding tests with the same feeds. From their data they have concluded that the group feeding technique, while it lends confirmation to the individual tests, may be of very limited value, due to its lack of sensitiveness, especially when the expected differences to be investigated are small.

It may be argued that differences too small to be detected by the group feeding method may be too small to have any practical significance; nevertheless, the individual feeding method has certain outstanding advantages. The quantity of feed consumed by each pig is known as is its effect on the individual, and at least an attempt can be made to correlate differences in individual performance with food consumption. Such differences may be brought out by the group feeding

method, but they may be so obscured by the variability of the individual response among the various group members, that definite assignment of them to feed is impossible. Thus the group feeding method is to be regarded as a link between practical feeding and scientific experimentation and as a means of verification under practical conditions of the individual feeding trial.

The present experiments yield some interesting data on the quantities of feed required per 100 pounds of body increase. In these experiments the average figure for this value is of the order of 285 pounds, the range being from 447 pounds on the 10% protein level (experiment 1) to 229 pounds on the 17% protein level (experiment 3) at body weights from 70 to 156 pounds. These figures give some idea of the efficiency with which feed is converted into body tissue and, on the whole, are much lower than those usually encountered in either group feeding work or under practical feeding conditions. While no definite average figure is available for actual practical conditions with efficient feeding, approximately 400 pounds of concentrates are usually required to produce 100 pounds of body gain. Less than this quantity of feed may be required in many cases but feed requirements below 300 pounds are unusual in general feeding practice.

The system of feeding used in these experiments has consistently given results for feed efficiency of the order herein reported and at the same time the rate of growth has been maintained satisfactorily. It is recognized that in the effort to maintain an equalized feed intake between three pigs, the feed intake of two members of the triplet was limited by that of the third pig, and that in a sense, limited feeding was being practiced on these two pigs. Under such conditions it is apparent that the efficiency of food conversion might have been almost at a maximum, there being no waste and no feed consumed merely in satisfaction of appetite.

Woodman and associates ('36) observed greater thriftiness among pigs fed individually as compared to those fed in groups, the former making the greater gains in the least number of

days. These authors suggest that there is a considerable saving of energy when pigs are individually fed as compared to group-fed pigs which by nature use up considerable energy in consequence of "scrambling" at feeding time at the common trough. The logic of their deductions is recognized and this point together with the possibility of a limited feed intake may explain the high efficiency of food conversion obtained in these experiments.

It should be further pointed out, however, that the average daily gains in experiment 2 exceeded those of experiment 3 in practically all instances, a result which may be due to starting the pigs on experiment 3 at a lighter weight (40 pounds) rather than at 80 pounds as in experiment 2. The resulting initial growth of the pigs on experiment 2 was slow due possibly either to an unrecognized deficiency of the ration or to limited initial reserves of some of the pigs.

SUMMARY

The optimum quantity of protein in the ration for pigs for the growth span from 40 to 210 pounds was found to be from 15 to 17%. Levels of protein of 20, 22, 25 and 27% were not outstanding in their superiority to the 15 and 17% levels, but the trend of the results favored the higher levels of protein intake for pigs from weaning up to a weight of approximately 75 pounds. The lower levels of protein intake were superior to the higher levels for pigs between the weights of 120 and 200 pounds. Rations containing 10 and 12% of protein were unsatisfactory.

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THE EFFECT OF CERTAIN CARCINOGENS ON VITAMIN A IN THE LIVER [1]

C. A. BAUMANN, E. G. FOSTER AND P. S. LAVIK

Department of Biochemistry, College of Agriculture, University of Wisconsin,
Madison

Several observations suggest that carcinogenic compounds may alter vitamin A metabolism. The intraperitoneal injection of dibenzanthracene into rabbits reduced the vitamin A content of liver mitochondria, while p-aminoazobenzene, a noncarcinogenic ring compound, was without effect (Goerner, '38). Livers of rats fed aminoazotoluene until hepatomas appeared contained less vitamin A than normal livers, and the tumors themselves were devoid of the vitamin (Goerner and Goerner, '39 a). Rats and rabbits injected intraperitoneally with dibenzanthracene showed markedly lowered hepatic stores of vitamin A as compared to uninjected controls, although the hepatic stores of vitamins C and D, and of glutathione were unaffected by the injections (Goerner and Goerner, '39 b, c).

Unfortunately, the material used in the latter experiments was commercial dibenzanthracene, a product which until recently has been quite impure (Fieser, '38). Furthermore, the animals' source of vitamin A was a commercial chow fed ad libitum, and the food consumption of the various groups was not recorded. It is well-known that the hepatic storage of vitamin A parallels the amount of vitamin ingested, and preliminary experiments of our own have indicated that the presence of an azo dye in certain rations may reduce food intake

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by as much as 40%. The intraperitoneal injection of hydro-carbons also lowered food consumption somewhat. Much of the observed reduction in vitamin A storage may therefore have been merely the effect of a reduced food intake. Inasmuch as very little is known about factors which alter the fate of vitamin A in the body, it became desirable to determine whether dibenzanthracene could affect hepatic storage of vitamin A under suitably controlled conditions, and if so, whether this phenomenon is related to carcinogenesis.

METHODS

Rats 3 to 4 weeks of age and 35 to 50 gm. in weight were used for the experiments. They were fed a basal ration devoid of vitamin A but complete in other dietary essentials. It had the following percentage composition: casein 18, salts 4, yeast 7.9, irradiated yeast 0.1, agar 2, cottonseed oil 5, dextrinized corn starch 63. On this ration growth ceases, and ophthalmic symptoms appear in 5 weeks.

The carcinogenic agents used were dibenzanthracene, methyl cholanthrene, benzpyrene, and butter yellow (dimethylamino-azobenzene). As examples of non-carcinogenic substances 1, 2 benzanthracene, and India ink were tested. In our earlier experiments the dibenzanthracene was purified by treatment with lead tetraacetate (Fieser and Hershberg, '38) and the methyl cholanthrene by chromatographic adsorption on Al_2O_3. The resulting products were white crystals and pale yellow crystals, respectively. Commercial samples of these agents obtained later were sufficiently pure for use directly.

Colloids for injection were prepared by dissolving the agent in hot acetone and adding it dropwise to an aqueous solution of gelatin maintained at 80–90°C. The residual acetone was distilled off under suction, and the final volume so adjusted that the colloid contained 1 mg. of hydrocarbon in 1 cc. of 1% gelatin. The samples were stored in the refrigerator when not in use. Hepatic vitamin A was determined colorimetrically by the $SbCl_3$ reaction (Carr and Price, '26) and the results expressed as blue units (Moore, '30) per liver.

EXPERIMENTAL

Rate of depletion of hepatic vitamin A

In the first experiment twenty rats 35 to 50 gm. in weight were fed the basal low A ration, plus 1 drop of halibut liver oil per rat daily for 6 days. At this point the supplement of halibut liver oil was discontinued. The oil contained 1250 blue units per drop. Two rats were killed on the seventh day and analysis revealed the presence of 2955 blue units of vitamin A per liver. The surviving animals were divided into three groups. The first received three intraperitoneal injections per week of 1 mg. each of colloidal dibenzanthracene, the amount used by Goerner and Goerner ('39 b). Since this is more than need be given to induce tumors in rats, a second group was injected with 1 mg. of dibenzanthracene once a week. The third group received no injections. The animals were kept on the low A diet and were killed for analysis at 3, 8 and 10 weeks. The results are summarized in table 1.

TABLE 1

The effect of intraperitoneal injections of colloidal dibenzanthracene on the rate of depletion of hepatic vitamin A [1]

Blue units per liver

WEEKS	UNINJECTED CONTROLS	DIBENZANTHRACENE INJECTION	
		3 mg./week	1 mg./week
0	2955 (2920–2990)	2955	2955
3	3155 (3120–3190)	2300	....
8	2420 (2330–2510)	703 (584–823)	520 (473–567)
10	1267 (1180–1317)	236 (105–368)	268 (258–278)

[1] Each rat received 1250 blue units per day for 6 days previous to beginning of administration of the hydrocarbon.

Marked differences between groups were observed in the rate of depletion of hepatic vitamin A. After 8 weeks the uninjected animals still had 2400 units of vitamin A in their livers, a loss of 600 units, whereas the two groups of animals treated with dibenzanthracene had 500 and 700 units, respectively, a loss of 2300 to 2500 units. The rate of depletion of vitamin A, therefore, had been increased approximately

fourfold by the dibenzanthracene. Since the rate of depletion of the vitamin was slightly greater in animals injected once weekly than in those injected thrice weekly, 1 mg. of dibenzanthracene per week was ample to demonstrate the effect. On this level whitish strongly fluorescent residues of the hydrocarbon were invariably found in the abdominal cavity on autopsy.

Animals injected thrice weekly contained much larger residues and in addition adhesions between the liver and adjacent tissues were present. Such animals were usually very emaciated, although even with the largest injections, growth continued during the early weeks of the experiment. At 8 weeks the average weights were 193 gm. for the uninjected control animals, 139 for those receiving 1 mg. dibenzanthracene per week, and 128 for those receiving 3 mg. per week. To reduce possible complicating effects of adhesions, a standard amount of hydrocarbon, viz., 1 mg., was injected weekly in all subsequent experiments.

Absorption and storage of vitamin A

Having established that dibenzanthracene affected the mechanisms by which vitamin A leaves the liver, an attempt was made to determine whether the hydrocarbon affected the mechanisms by which vitamin A enters the liver. The following procedure was adopted: eight rats, 50 gm. in weight, were divided into two groups of four and given the low A ration. The first served as uninjected controls, whereas the second received three intraperitoneal injections of 1 mg. colloidal dibenzanthracene during 1 week. Two days after the last injection the animals of both groups were fed 4 drops of halibut liver oil each, and 2 days later still, they were killed for analysis. No abnormalities were noted in any of the animals, except for the presence of hydrocarbon in those injected. The presence of hydrocarbon markedly depressed hepatic storage. Control livers contained 2840 to 3150 blue units per liver, the average being 3040 units; livers from injected animals ranged from 1260 to 1650, with an average of 1500 units.

Since our 50-gm. animals contain but 50 to 75 blue units per liver, the above discrepancy could not represent a loss sustained during the week of injection.

Furthermore, the rate of depletion of vitamin A from livers containing 3000 units ranges from 100 to 300 units per week (table 1) rather than the 1500 units of loss per week which would have to be assumed if the effect of the dibenzanthracene were attributed only to the mechanism of depletion. It therefore appears that the injected vitamin A had also interfered with the process by which vitamin A enters the liver.

Comparison of dibenzanthracene and methyl cholanthrene

Dibenzanthracene is a relatively weak carcinogenic substance; methyl cholanthrene, one of the most potent carcinogenic agents known. If the effect of dibenzanthracene on vitamin A metabolism were related to tumor formation, then the more potent carcinogen, methyl cholanthrene, might be expected to show an even greater effect on hepatic vitamin A. The two hydrocarbons were therefore compared for their over-all effect on vitamin A metabolism. Three groups of seven rats each, 40 gm. in weight, were placed on the low A diet and fed halibut oil by dropper. The oil was diluted with cottonseed oil so that 1 drop contained 200 blue units of vitamin A, and each rat was fed 3 drops per week throughout the experiment. The first group received no injections; the second received 1 mg. colloidal dibenzanthracene intraperitoneally once a week; the third received 1 mg. colloidal methyl cholanthrene once a week. The animals were killed for analysis at intervals. All animals grew well and no abnormalities appeared at autopsy. The results are summarized in table 2. Although both hydrocarbons reduced vitamin A stores, methyl cholanthrene caused less rather than more depletion than benzanthracene. Considering the animals killed at different times collectively, the uninjected controls averaged 1887 blue units per liver, the methyl cholanthrene animals averaged 892 and the dibenz-

anthracene animals 489 blue units per liver. Hence, dibenzanthracene was about twice as effective in reducing hepatic vitamin A as the more potent carcinogen, methyl cholanthrene.

TABLE 2

The action of methyl cholanthrene and of dibenzanthracene on hepatic vitamin A [1]

Blue units per liver

WEEKS OF EXPERIMENT	UNINJECTED CONTROLS	DIBENZANTHRACENE	METHYL CHOLANTHRENE
$4\frac{1}{2}$	1551	546	922
5	1452	462	953
$5\frac{1}{2}$	1980	410	850
			670 [2]
6	2310		760 [2]
	1782	861	1250 [2]
$6\frac{1}{2}$	2250	300	840
		345	
		498	
Average	1887	489	892

[1] Six hundred blue units of vitamin A were given to all animals weekly. The animals averaged 75 blue units of vitamin A per liver at the beginning of the experiment.

[2] Animals died.

Effect of other compounds

The effect of other compounds on hepatic vitamin A was next investigated. Young·rats 50 to 60 gm. in weight were placed on the low A ration, fed 6 drops of halibut liver oil during the first 6 days, and then continued on the low A ration. Except for group 6 they were injected intraperitoneally once a week with 1 cc. of colloidal carcinogen, and were killed for analysis at 8 weeks. Group 6 received 1 cc. of colloidal dibenzanthracene every other week, and was likewise killed after 8 weeks. The compounds, numbers of injections, numbers of animals, and the vitamin A content of the livers after 8 weeks are listed in table 3.

Colloidal butter yellow, dimethylaminoazobenzene, was practically devoid of any effect on hepatic vitamin A. This compound is of particular interest because its carcinogenic

action does not occur at the site of administration, like the hydrocarbons, but whether fed or injected it produces tumors in the liver instead (Kinosita, '40). Its failure to affect liver stores of vitamin A would appear to indicate that the production of hepatomata, and the storage and unloading of hepatic vitamin A are distinct and unrelated processes, even though they both occur in the same organ.

TABLE 3

The effect of various carcinogenic agents on the hepatic vitamin A content of rats maintained for 8 weeks on a low A diet

COMPOUND	NUMBER OF INJECTIONS	NUMBER OF ANIMALS	RANGE: HEPATIC VITAMIN A	AVERAGE BLUE UNITS/LIVER
			blue units	
Control	0	4	3100–4120	3700
Butter yellow	8	4	2200–4300	3425
Benzpyrene	8	4	2460–3530	2990
Benzanthracene [1]	8	4	1800–3600	2760
Methyl cholanthrene	8	6	1600–2800	2100
Dibenzanthracene	4	4	1740–3000	2340
Dibenzanthracene	8	3	1440–1560	1490

[1] This compound is non-carcinogenic, although related chemically to many carcinogens.

Benzpyrene and 1, 2 benzanthracene accelerated the depletion of hepatic vitamin A to about the same extent. Livers of animals receiving these compounds contained 2990 and 2760 blue units of vitamin A respectively, as compared to 3700 for the uninjected controls (table 3). This similarity is particularly striking in view of the very great differences in the tumor-producing power of the two hydrocarbons, benzpyrene being a potent carcinogen whereas 1, 2 benzanthracene is practically devoid of carcinogenic activity (Fieser, '38). Evidently, therefore, the effect of a hydrocarbon on vitamin A does not necessarily parallel its carcinogenic potency.

Animals receiving eight injections of methyl cholanthrene contained 2100 blue units per liver; those receiving four injections of dibenzanthracene contained 2340 (table 3). In other words, about twice as much methyl cholanthrene as

dibenzanthracene was needed to produce a comparable effect on vitamin A storage, again illustrating the discrepancy between carcinogenic potency and effect on hepatic vitamin A. Animals receiving eight injections of dibenzanthracene had the lowest stores of all, 1490 blue units. Incidentally in another series it was shown that colloidal carbon black (India ink diluted 1:8 with water) was without effect on hepatic vitamin A.

The effects of the compounds on hepatic vitamin A appear to be metabolic. They are not artifacts of the chemical determination, since the addition of any of the hydrocarbons, or of butter yellow, to the $SbCl_3$ reaction mixture failed to alter the intensity of the blue color produced. In seventeen experiments various hydrocarbons were added to minced liver, incubated for periods of time up to 4 hours, and the tissue digested and analyzed as before. In no case did samples treated with hydrocarbon differ in vitamin A content from comparable aliquots free of hydrocarbon.

Effect of carcinogenic amounts of hydrocarbons

In all previous experiments hydrocarbons were injected intraperitoneally because this was the method used by Goerner ('38), and Goerner and Goerner ('39 a, b, c) and because the technique readily produced changes in hepatic vitamin A. However, this is not the usual method employed in studying tumor production with these agents. No tumors appeared during the studies on vitamin A storage because the experiments lasted for only 10 weeks or less, whereas at least 4 months, and sometimes as much as a year, are needed to produce tumors in rats with these hydrocarbons (Fieser, '38; Rusch, Baumann and Maison, '40). A "carcinogenic method" of administering the hydrocarbon was therefore employed, and the effect on vitamin A storage determined.

Methyl cholanthrene and dibenzanthracene were dissolved in lard (8 mg./cc.) and ½ cc. was injected subcutaneously, twice the first month, and once monthly thereafter. Three groups of ten rats each were used. One group received lard

only; the others methyl cholanthrene and dibenzanthracene respectively. The animals averaged 65 gm. in weight at the beginning of the experiment when they were placed on the low A diet and given 2 drops of halibut liver oil daily for 3 weeks. Thus enough hepatic vitamin A was accumulated to supply the needs of the animals until tumors appeared. Animals were killed for analysis at intervals. After 18 weeks, all of the survivors treated with methyl cholanthrene had palpable tumors,[2] the largest being 4×5 cm. in diameter. No tumors appeared in the other groups.

The livers of animals killed during the nineteenth week contained the following amounts of vitamin A:

Controls	3250 blue units, range 1710–5570
Methyl cholanthrene	2500 blue units, range 2165–2810
Dibenzanthracene	1320 blue units, range 1150–1530

In view of both the large amounts of vitamin A originally administered and the long depletion period, wide variations within groups might be expected. Nevertheless, several conclusions are warranted by the data: (1) dibenzanthracene lowers hepatic vitamin A whether the hydrocarbon is given subcutaneously in oil or intraperitoneally as a colloid; (2) given in oil, dibenzanthracene exerts more effect on vitamin A than methyl cholanthrene; (3) even the actual presence of tumors due to methyl cholanthrene failed to produce any very considerable reduction in the liver stores of the vitamin.

Effect of spontaneous tumors on vitamin A stores

Our strain of rats is subject to the development of spontaneous mammary adenofibromas.[2] These tumors are slow-growing, encapsulated, and frequently attain an enormous size without either invading the body wall or becoming necrotic. The tumor contains much connective tissue. Analyses of the livers of adult rats bearing tumors which weighed from 7

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[2] We are indebted to Dr. H. P. Rusch of the McArdle Memorial Laboratory, University of Wisconsin, for the examination of these tumors.

to 100 gm., showed a range of 7000 to 17,000 blue units of vitamin A per liver, average 12,800. The variations could not be related to tumor size. Analyses of fourteen livers from stock rats of similar age showed ranges of 4500 to 13,100 blue units per liver, average of 8200 blue units. The latter figure is somewhat greater than the result of a survey made 7 years ago, when the average in the colony was found to be 6000 blue units (Baumann, Riising and Steenbock, '34).

The presence of even a very large tumor, therefore, does not result in a reduction of hepatic vitamin A, as experiments with dibenzanthracene might lead one to suppose. On the contrary, the stores may actually be increased if the tumor-bearing rats consume more food and hence more vitamin A. However, a diet low in vitamin A has been reported to cause a benign type of growth, odontoma, in rat incisors (Orten, Burn and Smith, '37), although low A diets did not affect tumor production due to benzpyrene (Howe, Elliot and Shear, '40). Sure, Buchanan and Thatcher ('36) have shown that enormous doses of carotene do not affect tumor growth.

Deficiency symptoms

The effectiveness of dibenzanthracene in reducing liver stores of vitamin A suggested that the hydrocarbon might hasten the appearance of deficiency symptoms. This proved to be the case with two groups of twelve rats each, which were placed on the low A diet when 40 to 45 gm. in weight, the rats of one group being injected with 1 mg. dibenzanthracene weekly, and the others serving as controls. Ophthalmia appeared in the injected animals on an average of 3 days sooner than in those not injected, and the growing period was shortened by 9 days (30 as compared to 39). The rate of growth was markedly retarded by the hydrocarbon, a phenomenon which, however, is also observed when adequate amounts of vitamin A are present. Attempts at comparing the vaginal smear responses (Baumann and Steenbock, '32) were unsuccessful, because the vaginal orifice of the injected animals failed to open.

DISCUSSION

Whatever the explanation or the mechanism involved, it is evident that numerous hydrocarbons tend to reduce the storage of vitamin A in the liver, and furthermore, that the dibenzanthracene molecule possesses this property to a very high degree. The phenomenon is apparently unrelated to tumor formation, in spite of the fact that certain compounds (DBA, MC, BP) are active both as carcinogens and as "anti-storage agents." However, 1, 2 benzanthracene is an example of a compound which reduces vitamin A storage without being carcinogenic, whereas butter yellow is a carcinogen which does not reduce hepatic vitamin A. The situation might be compared to the relationship between certain estrogens and carcinogens. Here too, some molecules, such as benzpyrene and estrone, possess both properties, but their activities do not run parallel, and examples can be cited of compounds which show only the one or the other activity (Cook et al., '34; Lacassagne, '36).

In general, most recorded attempts at lowering hepatic vitamin A by physiological techniques have been unsuccessful unless the absorption of the vitamin was disturbed. Thus, vitamin A storage is normal in many clinical disorders (Moore, '37; Ellison and Moore, '37; Wolff, '32) as well as in the experimental loss of liver fat due to starvation or vitamin B_1 deficiency (Dann and Moore, '31), or the choline-induced loss of liver fat (Lease and Steenbock, '39). Liver damage due to phosphorus, $CHCl_3$ or CCl_4 apparently does not alter vitamin A storage (Lasch, '35; Wendt and Koenig, '37; Greaves and Schmidt, '35, '36). Furthermore, the alleged antagonism between vitamin A and thyroxin has not been universally accepted (Logoras and Drummond, '38; Baumann and Moore, '39). On the other hand, reduced storage of vitamin A has been observed when the reticulo-endothelial system was blocked with thorotrast (Lasch and Roller, '36), phenylhydrazine, or bismuth (Wendt and Koenig, '37). Alcohol, likewise, is said to reduce the storage of vitamin A

(Ikegaki, '38). It is to these latter substances that the action of the hydrocarbons should be compared, even though the mechanism involved is as yet unknown.

SUMMARY

1. Colloidal dibenzanthracene, injected intraperitoneally, increased the rate of depletion of hepatic vitamin A fourfold, and also appeared to interfere with the entry of vitamin A to the liver. Dibenzanthracene injected subcutaneously in oil also increased vitamin A depletion.

2. Methyl cholanthrene and benzpyrene, two potent carcinogens, and 1, 2 benzanthracene, a non-carcinogenic hydrocarbon, likewise reduced hepatic vitamin A, but all three were quantitatively less effective than dibenzanthracene. There was therefore no correlation between the carcinogenicity of a compound and its effect on vitamin A. Butter yellow, which is carcinogenic, and carbon black, which is non-carcinogenic, were without effect on the vitamin.

3. The livers of rats with spontaneous tumors contained more vitamin A than the livers of non-tumorous controls. Livers of rats with tumors due to methyl cholanthrene contained more vitamin A than non-tumorous rats treated with dibenzanthracene. Decreased vitamin A, therefore, is not a necessary prerequisite to tumor formation.

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THE SPARING ACTION OF THIAMINE ON BODY TISSUE CATABOLISM [1]

BARNETT SURE AND MAURICE DICHEK

Department of Agricultural Chemistry, University of Arkansas, Fayetteville

During 1931–1932 this laboratory published a series of papers in which data were presented, showing that vitamin B_1 exerts a per se influence on growth, as evidenced by the paired-feeding method of experimentation. Such conclusion is in accord with that of Mitchell ('33) and with those of Graham and Griffith ('33), but it is at variance with those of Hogan and Pilcher ('33) and McClure, Voris and Forbes ('34). Since in the years 1931 to 1934 vitamin B concentrates were employed as a source of thiamine, it is possible that the former produced an influence on growth by furnishing several components of the vitamin B complex. The earlier work, therefore, needed reinvestigation; consequently new experiments were carried out, using the technique previously described (Sure and associates, '31 and '32) with the following modifications. The feeder employed was that introduced by Swift, Kahlenberg, Voris and Forbes ('34) which we had constructed with heavy wire prongs to discourage animals from sitting on the lid of the feeder or from attempting to crawl inside the feeding cup. As the animals grew we found it necessary to increase the size of the feeder. The food was weighed every other day on an agate bearing beam balance to an accuracy of 10 mg., and transferred to the food containers,

according to the technique of Hogan and Pilcher ('33). No difficulties were encountered with this type of feeder in obtaining accurate quantitative records of food consumption. Any small particles of food spilled on the platform of the feeder were carefully transferred to the cup with the aid of a fine brush.

EXPERIMENTAL

The results of this investigation are presented in tables 1 and 2. The experiments were carried out in four series.

Series I. This study was conducted on ten pairs of rats subsisting on a purified diet with the following percentage composition: casein (vitamin-free)[2] 18; salt mixture no. 351 (Hubbell, Mendel and Wakeman, '37) 4; agar-agar 2; nicotinic acid 0.05; hydrogenated cottonseed oil[3] 10; dextrin 65.95. This was supplemented daily with 20 µg. riboflavin, 20 µg. pyridoxin, 6 mg. choline, and 0.2 cc. to 0.5 cc. of a solution containing the "W" factor, equivalent to 0.2 and 0.5 gm. liver extracts respectively. The "W" factor extracts provided 30 to 75 µg. pantothenic acid daily as well as other unidentified filtrate factors essential for growth. The control animals received in addition 20 µg. thiamine daily. Each animal received daily except Sundays 6 drops of cod liver oil as a source of vitamins A and D. On such a diet fed to control animals ad libitum excellent growth was secured; but when the control rats were restricted to the diminishing food intakes of the thiamine deficient animals, during an average experimental period of 50 days, they lost an average of 4.5 gm. per rat. The thiamine deficient animals, however, lost an average of 19.1 gm. per rat, or over 400% more than the litter mate controls. In other words, the thiamine administered to the control animals produced a pronounced sparing effect on body tissue catabolism (table 1).

[2] Supplied by the Borden Company, New York, under the trade name of "Labco."

[3] Crisco.

TABLE 1

The role of thiamine in body tissue catabolism (ration 11)

PAIR NUMBER [1]	PERIOD OF EXPERIMENTATION	INITIAL WEIGHT	FINAL WEIGHT	CHANGE IN WEIGHT
	days	*gm.*	*gm.*	*gm.*
1 — P	58	95	87	—8
RC		98	89	—9
2 — P	57	86	83	—3
RC		90	91	+1
3 — P	36	84	62	—22
RC		87	67	—20
4 — P	48	96	70	—26
RC		87	83	—4
5 — P	62	95	76	—19
RC		95	78	—17
6 — P	52	106	71	—35
RC		104	96	—8
7 — P	54	86	84	—2
RC		90	108	+18
8 — P	54	84	76	—8
RC		92	97	+5
9 — P	37	102	62	—40
RC		98	86	—8
10 — P	42	88	60	—28
RC		84	81	—3
Average change in weight per group during an average experimental period of 50 days.			P	—19.1
			RC	—4.5

[1] P = pathological; RC = restricted control. Restricted control animals received 20 µg. B₁ daily.

Series II. These experiments, as well as those of series III and IV, were conducted on ration 9 which has the following percentage composition: casein,[4] 16; salts (Hubbell, Mendel and Wakeman, '37), 4; autoclaved baker's yeast, 7; autoclaved beef, 8; hydrogenated cottonseed oil, 9; and dextrin, 56. Realizing, however, that riboflavin and pyridoxin may also suffer some destruction during autoclaving, in a number of experiments, either riboflavin or pyridoxin or both were given in supplementary doses of 10 µg. to the pathological and control animals. The control rats, in addition to receiving

[4] Thoroughly extracted with acidulated water and dilute ethyl alcohol.

every other day the same amount of food as the litter mates consumed the previous 48 hours, were given separately from the ration 20 µg. thiamine daily in a diluted solution in caster cups. Each animal received daily except Sundays 6 drops of cod liver oil as a source of vitamins A and D. Fourteen pairs of animals were used and the average experimental period was 42 days. Five pathological animals in this series developed marked convulsions during the terminal stages of the thiamine avitaminosis. During this stage there was an onset of marked anorexia and the body tissue catabolism in the polyneuritic animals was much greater than in the litter mate controls. To conserve space, detailed data are deleted. Further evidence on the role of thiamine in its sparing action on body tissue catabolism in this deficiency disease is presented in table 2 which shows the results of series III experiments.

It has been our experience during the past 12 years that when yeast and beef or both are autoclaved at the natural pH of these products at 15 pounds pressure, frequently appreciable amounts of thiamine are left, as evidenced by the minute amounts of supplementary crystalline thiamine that are needed daily to produce continuous growth. For this reason, we now autoclave the baker's yeast as well as the beef at 20 instead of 15 pounds pressure.

Series III. The next procedure adopted was aimed at determining whether increased amounts of thiamine given as supplements to the same food intake will produce a greater economy of food utilization. For this investigation ration 9 was used because it had been found to require only minute amounts of supplementary thiamine to permit slow but continuous growth. The thiamine was administered in daily doses of 0.1 µg. to 1.0 µg. following maintenance until it was apparent that growth could not be checked even with the small daily doses. A vitamin depletion period was then begun. The thiamine was not given from the fiftieth to the fifty-eighth day of experimentation, which allowed a depletion period of about one-half of the entire experimental period. The pathological animals received a total ranging from 0.5 to 16.4 µg.

thiamine during the entire experimental period. Since the control animals received 20 μg. thiamine daily, they received during 50 to 58 days a total of 1000 to 1160 μg. thiamine during the same interval of study. Yet, when a comparison is made between initial and maximum weights of the control and pathological animals, no differences in growth are apparent. In other words, 70 to 2000 times the amounts of thiamine as supplements to the same daily food intake produced no greater economy in food utilization. When, however, during the second half of the experimental period the thiamine was withheld from all rats, all animals lost weight, but the losses were much more pronounced in those rats which received the small doses of thiamine than in the control litter mates which received much larger allowances of this vitamin. There was an average loss of over 45% more body weight in the pathological than in the control rats during the thiamine depletion period. During the latter period the food consumption decreased 50 to 100% which, of course, accounts for the body tissue catabolism, but it is quite clear from the results of the experiments of this series that the greater intake of thiamine exerted a pronounced influence in sparing tissue catabolism during periods of inanition produced by this vitamin deficiency.

The failure of McClure, Voris and Forbes ('34) to find a function for thiamine in the economy of food utilization can now be explained by the fact that, with their experimental procedure, the pathological animals received an occasional unit of thiamine, which allowed slow growth.

Series IV. The next procedure was designed to determine the daily dose of thiamine required to keep the pathological animals as near maintenance as possible on ration 9 (with baker's yeast of this ration autoclaved at 20 pounds instead of 15 pounds pressure) supplemented with daily allowances of 10 μg. riboflavin and 10 μg. pyridoxin. The control rats received in addition 20 μg. thiamine daily. It was determined that approximately 0.05 μg. thiamine daily was required. When, however, there was an indication of slow growth, the thiamine

TABLE 2

The role of thiamine in body tissue catabolism (ration 9 with minute amounts of thiamine to the pathological animals)

PAIR NUMBER [1]	PERIOD OF EXPERI-MENTATION	INITIAL WEIGHT	MAXIMUM WEIGHT	LOSS IN WEIGHT FOLLOWING THIAMINE DEPLETION	TOTAL THIAMINE GIVEN PATHOLOGICAL ANIMALS DURING ENTIRE EXPERIMENTAL PERIOD
	days	*gm.*	*gm.*	*gm.*	*micrograms*
25 — P	103	70	108	33	6.5
RC		68	104	12	
26 — P	114	54	158	46	1.8
RC		50	166	44	
27 — P	103	58	121	36	0.5
RC		55	130	20	
28 — P	103	56	122	28	0.6
RC		64	130	24	
29 — P	100	60	103	27	0.5
RC		56	110	14	
30 — P	103	58	128	33	0.6
RC		54	133	15	
31 — P	109	70	142	28	12.2
RC		67	127	23	
32 — P	104	75	170	47	16.4
RC		73	172	33	
33 — P	105	66	151	33	12.4
RC		68	160	26	
34 — P	94	59	132	38	8.9
RC		54	125	21	
35 — P	94	54	161	53	8.6
RC		50	172	44	

[1] P = pathological; RC = restricted control. Restricted control animals received 20 µg. B_1 daily.

was withheld, or if a definite decline in growth was threatened, the daily dose was temporarily increased to 0.1 µg. In seven pairs thus studied, so long as the deficient animals were kept at maintenance with minute amounts of thiamine, the control rat, which received 100 times the amount of this vitamin (on the same food intake) showed no greater growth; that is, at an approximately maintenance level, greater amounts of thiamine produced no influence on economy of food utilization.

Just how thiamine functions in its influence in sparing body tissue catabolism is not clear from the data submitted. It is now accepted that thiamine acts in the animal body as a catalyst, necessary for the degradation of pyruvate after it has been converted into its diphosphate ester, cocarboxylase (Banga, Ochoa and Peters, '39). Possibly its beneficial influence is associated with its role in intermediate carbohydrate metabolism. This is a problem that deserves further investigation.

SUMMARY

When inanition with its loss in body weight is produced by thiamine deficiency, greater intakes of this vitamin produce a sparing influence on body tissue catabolism. The mechanism of such action is not clear from the data submitted; it is suggested that this may be associated with the role thiamine plays in intermediate carbohydrate metabolism.

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RIBOFLAVIN AS A FACTOR IN ECONOMY OF FOOD UTILIZATION [1]

BARNETT SURE AND MAURICE DICHEK

WITH THE ASSISTANCE OF MARTIN M. CITRON

Department of Agricultural Chemistry, University of Arkansas, Fayetteville

Since we could find no evidence in the literature on the role of uncomplicated riboflavin deficiency in the economy of food utilization, a study was made of this problem, the results of which are submitted in this communication. The technique of animal feeding was the same as that described in the preceding paper (Sure and Dichek, '41). The study was conducted in two series of experiments on two types of diets. The first was ration 10, which is a modification of the riboflavin deficient diet of Day and Darby ('38), and has the following percentage composition: Casein,[2] 18; salts 351, 4;[3] hydrogenated cottonseed oil,[4] 8; cod liver oil, 2; and dextrin, 68. The dextrin carried an 80% alcoholic extract of 25 gm. rice polishings in 100 gm. of ration, as a source of all the components of the vitamin B complex with the exception of riboflavin. The control animals were restricted by 2-day intervals to the same ration and the same amount of food consumed by the riboflavin deficient litter mates, and in addition received 20 μg. crystalline riboflavin daily.

It was found early in this investigation that, after 40 to 50 days on ration 10, a pyridoxin anorexia develops. At a point

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[1] Research paper no. 673 Journal Series, University of Arkansas. This is paper XXI in the series of AVITAMINOSIS. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

[2] Thoroughly washed with acidulated water and dilute ethyl alcohol.

[3] Sure and Dichek ('41), page 446.

[4] Crisco.

when the riboflavin deficient animals are in a marked state of inanition the supplementary administration of 10 µg. pyridoxin daily to such pathological rats is accompanied by an increase in food consumption and growth. As a matter of fact, it was not possible to demonstrate the beneficial effect of riboflavin in economy of food utilization unless ration 10 was supplemented with pyridoxin, which was done in all the experiments of series I. It was thought that following the marked pyridoxin anorexia the decreased amount of food intake might not furnish a sufficiency of thiamine supplied by the rice polish extract in the ration; hence, this ration was supplemented for both the pathological and control animals with 10 µg. thiamine as well as 10 µg. pyridoxin daily.

The second series of experiments was carried out with ration 11, the composition of which has already been given (Sure and Dichek, '41). Ration 12 differed from 11 only in that it contained 0.1 instead of 0.05% nicotinic acid. Rations 11 and 12 were supplemented daily with 20 µg. thiamine, 20 µg. pyridoxin, 6 mg. choline, and 0.2 cc. of a concentrate of "W" factor, equivalent to 0.2 gm. of liver extracts as a source of the filtrate factors. The control animals, which were restricted to the same ration and same amount of food as the pathological litter mates, received in addition 20 µg. crystalline riboflavin daily. All animals in this investigation received 6 drops of cod liver oil, as a source of vitamins A and D, daily except Sundays.

The animals at initial weights of 40 to 70 gm. were taken from our stock colony which is fed our diet no. 2, the one introduced by Sherman and Muhlfield ('22). The fact that the vitamin deficient animals were able to make slow but continuous growth on ration 10 would indicate that the 80% alcoholic extracts from rice polishings provide appreciable amounts of riboflavin. Five pathological animals, however, out of eighteen died from riboflavin deficiency while subsisting on this ration. Fortunately the collapse occurred in the daytime so the final weight records are accurate. In the case of the animals on rations 11 and 12, sufficient riboflavin

for slow growth of the pathological rats must have been furnished by the "W" factor extracts, although such extracts were treated twice with fuller's earth at a pH of 3 to 4. The marked differences, however, in growth between the pathological and litter mate controls that received 20 μg. riboflavin daily are evident from tables 1 and 2.

At the termination of the experiments twenty pairs of rats selected from series I and II, were freed from their alimentary fills, ground through a sausage mill and dried to constant weight in an electric oven at a temperature of about 55°C. They were then extracted with ether in continuous Soxlet extractors for 24 hours. The ground fat-extracted rats were then reground in the sausage mill to a fine powder and redried at 103°C. for several hours during which procedure the small amounts of residual moisture were lost. One gram samples in duplicate were then taken for ash and total nitrogen determinations. The total nitrogen obtained multiplied by the factor 6.25 was taken as the protein in the sample. In addition, twenty-six rats from the same rat colony as the experimental animals were taken for analysis. Half of these were males and half females, with weights and ages the same as those of the animals of series I and II when they were started on experiments. The body carcasses of these animals were also freed from alimentary fills, dried and analyzed for fat, ash, and protein. The differences in chemical composition of the dry carcasses of these rats and the dry carcasses of the animals sacrificed at the termination of the experiments were taken as representing the fat, ash, and protein contents of the body gains. All the results of this investigation are presented in tables 1 and 2.

The experiments of series I (table 1) were performed on a total of eighteen pairs of animals, fourteen of which were male, and four female. The period of experimentation ranged from 50 to 188 days. The animals were given ration 10 supplemented with pyridoxin and thiamine as previously indicated. The "W" factor given in three cases for 16 to 34 days

was not found essential as a supplement of the vitamin B complex and was soon omitted.

The control animals that received the same ration and the same amount of food as the pathological rats, but in addition 20 µg. riboflavin daily, showed increased gains of 56 to 1300% over their riboflavin deficient litter mates. Although the period of experimentation differed for the various pairs of rats, the treatment of the control rats was in every case the same as that of their respective litter mates except for the fact that the former received riboflavin supplements. It, therefore, seems justifiable to present average gains per animal in this as well as the second series. In series I the average gain in body weight per animal was 43.8 gm., and for the control animals 82.2 gm. The pronounced influence of riboflavin in economy of food utilization for synthesis of body tissue is then most evident.

In series II there were six pairs of females and nine pairs of males maintained on rations 11 and 12, in which the components of the vitamin B complex with the exception of the filtrate factors were furnished in the form of crystalline vitamins. The filtrate factors were supplied by the "W" extracts prepared according to the procedure of Frost and Elvehjem ('37). The results in this series are also consistent, fourteen out of the fifteen pairs showing larger gains in weight in the control animals compared with the riboflavin deficient litter mates. The average gain in weight per animal for the riboflavin deficient animals was 56.0 gm. and for the litter mate controls on the same ration and same amount of food, 91.0 gm. Since the results of series II experiments are very similar to those of series I, detailed data are not presented.

The influence of riboflavin deficiency on chemical composition of the bodies was determined on twenty pairs of animals, but to conserve space, such figures are omitted. These figures were, however, used in calculations of chemical composition of body gains which are presented in table 2. There were no noteworthy differences in moisture content between the ribo-

TABLE 1

The role of riboflavin in economy of food utilization (series I, ration 10)

PAIR NUMBER [1]	PERIOD OF EXPERI- MENTATION	BODY WEIGHT		CHANGE IN WEIGHT
		Initial	Final	
	days	*gm.*	*gm.*	*gm.*
1 — P [2]	94	53	90	+37
RC	94	50	146	+96
2 — P	50	44	51	+7
RC	50	47	68	+21
3 — P	122	39	100	+61
RC	122	38	133	+95
4 — P	101	40	56	+16
RC	101	38	91	+53
5 — P	108	38	68	+30
RC	108	38	96	+58
6 — P	79	40	78	+38
RC	79	40	100	+60
7 — P	155	38	147	+109
RC	155	38	188	+150
8 — P	156	38	82	+44
RC	156	38	154	+116
9 — P	98	36	64	+28
RC	98	38	114	+76
10 — P [2]	72	38	36	—2
RC	72	38	55	+17
11 — P [2]	50	34	36	+2
RC	50	37	63	+26
12 — P	77	40	38	—2
RC	77	40	66	+26
13 — P	78	49	66	+17
RC	78	49	108	+59
14 — P [2]	72	44	54	+10
RC	72	38	67	+29
15 — P	181	38	162	+124
RC	181	38	233	+195
16 — P [2]	78	52	57	+5
RC	78	53	78	+25
17 — P	188	48	165	+117
RC	188	47	228	+181
18 — P	185	44	192	+148
RC	185	44	240	+196

[1] P = pathological; RC = restricted control.
[2] Animal died.

flavin deficient and control rats. From table 2 it is apparent that the greatest difference in chemical composition of body gains between the riboflavin deficient and the control rats was in fat, the control rats having gained eighty to several hundred

times more in fat than the litter mate pathological animals. The control rats also showed considerably more gain in protein than the pathological animals but the changes in ash are too small and variable to be of any significance.

TABLE 2

Influence of riboflavin deficiency on changes in chemical composition of body gains

RATION NUMBER	PAIR NUMBER [1]	PERIOD OF EXPERIMENTATION	CHANGE IN BODY WEIGHT	CHEMICAL COMPOSITION OF BODY GAIN		
				Fat	Ash	Protein
		days	*gm.*	*gm.*	*gm.*	*gm.*
11	21 — P	92	+34	—3.17	+2.59	+9.95
	RC		+79	+3.99	+4.53	+20.28
11	19 — P	135	+79	+9.56	+3.32	+16.80
	RC		+103	+14.42	+6.12	+25.36
11	26 — P	136	+61	+7.82	+3.53	+12.41
	RC		+98	+9.88	+4.67	+20.94
11	28 — P	127	+88	+7.11	+4.79	+19.64
	RC		+141	+13.31	+5.63	+30.98
11	23 — P	80	+12	—3.85	+2.43	+5.11
	RC		+52	—0.40	+3.16	+14.66
11	25 — P	72	+31	+2.69	+1.93	+11.47
	RC		+38	+4.88	+1.79	+11.91
11	29 — P	179	+50	+1.44	+3.82	+13.32
	RC		+80	+6.33	+4.24	+18.93
11	32 — P	219	+74	+4.49	+4.23	+16.41
	RC		+132	+22.72	+5.59	+25.35
12	31 — P	153	+94	+3.64	+5.35	+21.90
	RC		+134	+21.57	+6.02	+28.91
11	33 — P	212	+120	+7.88	+6.68	+24.07
	RC		+153	+16.17	+8.57	+30.13
10	2 — P	50	+7	—2.86	+1.45	+3.90
	RC		+21	+0.43	+1.28	+6.11
10	9 — P	98	+28	—3.43	+3.01	+5.08
	RC		+76	+3.85	+2.66	+13.97
10	7 — P	156	+44	+0.47	+3.20	+12.00
	RC		+116	+19.40	+5.37	+30.21
10	11 — P	72	+2	—3.34	+0.78	+0.71
	RC		+26	+0.35	+1.64	+7.25
10	13 — P	78	+17	—3.56	+2.03	+5.03
	RC		+59	+4.24	+3.25	+15.98
10	15 — P	181	+124	+10.33	+5.69	+23.80
	RC		+195	+20.25	+8.44	+45.58
10	17 — P	188	+117	+8.61	+6.27	+23.38
	RC		+181	+30.28	+7.62	+36.06
10	18 — P	185	+148	+7.82	+7.37	+39.21
	RC		+196	+36.12	+8.27	+37.57
10	6 — P	79	+38	+2.25	+1.49	+6.45
	RC		+60	+3.74	+4.05	+14.40
10	4 — P	101	+16	—2.18	+1.60	+4.30
	RC		+53	+0.27	+3.06	+12.62

[1] P = pathological; RC = restricted control.

DISCUSSION

The only investigation that approximates ours reported in the literature is the one by Braman and associates ('35). These investigators reported on twelve pairs of rats studied by the paired-feeding method and subsisting on diets deficient in vitamin G for a period of 14 weeks. Considerable difficulties were experienced in keeping their vitamin G deficient animals alive and frequently small amounts of yeast were given. The fact that the pathological rats developed dermatitis would indicate that there was also a deficiency of the acrodynia factor, pyridoxin. The control animals received an aqueous extract of Lilly's liver concentrate no. 343, as a source of vitamin G following the seventh week; previously dry-heated yeast was used. The average gain in body weight per animal was 25.9 gm. for the vitamin G deficient animals and 41.3 gm. gain for the controls, or an increase in 59.4% representing economy of food utilization for the animals receiving the vitamin G supplement. The chemical composition of the body gains showed 0.6 gm. of fat and 1.22 gm. nitrogen for the pathological animals and 3.1 gm. of fat and 1.74 gm. nitrogen for the controls. It is interesting to observe that, although these investigators were handicapped in 1935 in the construction of an uncomplicated riboflavin deficient diet, the relative differences they found in gains of body fat in their vitamin G deficient and control animals compare strikingly with the differences in gains of fat found in our riboflavin deficient and control animals. The authors, however, state: "That liver contains an abundance of vitamin G has been quite established, and there is evidence that it may also contain other factors especially favorable to growth and reproduction. It is recognized, therefore, that the water extract of the liver concentrate used in this experiment may have furnished more than a single factor in exerting its influence on the utilization of the protein and energy of the food." On the other hand, from the results of our investigation, it is quite clear that we were concerned with only one limiting factor, namely, riboflavin deficiency.

SUMMARY

Riboflavin produces a most pronounced effect on economy of food utilization for synthesis of body tissues. The increases in body gains were derived mainly from fats and to a lesser but appreciable extent from proteins.

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VITAMIN A DEFICIENCY: A FIELD STUDY IN NEWFOUNDLAND AND LABRADOR

DAVID STEVEN AND GEORGE WALD [1]

Biological Laboratories of Harvard University, Cambridge, Massachusetts

ONE FIGURE

For the past 3 years this laboratory, with others, has studied the course and cure of night-blindness in experimental vitamin A deficiency (Wald, Jeghers and Arminio, '38; Wald and Steven, '39). In order to apply and extend this work, we undertook a field study in the region of Northern Newfoundland and Labrador served by the International Grenfell Mission. Beriberi is endemic in this area, and recurrent epidemics of night-blindness have been reported from it (Appleton, '21; Aykroyd, '30). The measurements were made by one of us (D. S.) from September, 1939, through May, 1940. They are concerned with the design and application of a visual method for the survey of vitamin A deficiency; and with a study of the mutual effects of vitamin A deficiency and beriberi upon the visual threshold.

I. THE MEASUREMENT OF VITAMIN A DEFICIENCY

A. Apparatus and procedure

For the measurement of visual threshold a portable adaptometer was designed by one of us (G. W.).[2] It weighs about 20 pounds, and is completely enclosed in a cabinet 8.5 × 8.5 ×

11.5 inches in size. It includes a test unit, resembling a pocket lamp in size and shape, which contains the test lamp, a shutter set at 1/50 second, and at its front the test field. On top of this unit a small housing containing a miniature lamp provides a red fixation point. A funnel-shaped eye-piece which attaches to the test unit sets the subject's head in the correct position and protects the eyes from stray light.

The test unit is connected with a long flexible cord to the cabinet, which contains storage cells, a voltmeter and rheostat for regulating the line voltage, and a calibrated rheostat with scale for controlling the brightness of the test patch. This can be varied over an effective range of about 1 to 1 million. It was calibrated in milli-microlamberts (10^{-6} millilamberts) with a Macbeth illuminometer. This instrument measures brightness for the human eye directly, and so circumvents the difficulty that a rheostat modifies the color of the source as well as its intensity. All brightnesses in the present paper are expressed logarithmically for reasons which have been discussed previously (Wald et al., '38).

The subject faces a circular test field which subtends an angle of 2.4 degrees with the eye, and which is centered 6 degrees below the fixation point. In performing a measurement the test field is set initially at a sub-threshold intensity. Between successive flashes the intensity is raised by roughly equal logarithmic steps (i.e., by a constant proportion), until the subject reports just seeing the flash. The scale reading and time in darkness are then noted.

Following the recommendation of Wald et al. ('38) we measured only the threshold of the completely dark adapted eye. This rises earlier and further in vitamin A deficiency than the threshold in any other state of adaptation. It maintains a constant level at each sitting and so permits repetition and averaging of the measurements. It is probably the most accurate single index of vitamin A deficiency now available.

Readings were made after a subject had been in complete darkness for at least 20 minutes, and when necessary were

continued until a constant level was attained. Usually several persons were dark adapted simultaneously. As each subject left the dark room another entered. In this way, following the initial 20-minute interval, measurements could be continued without interruption. Tests on each individual were usually completed within 5 minutes.

Reliable measurements could not be obtained from children below 6 years of age. In the age group 6–8 years about one-quarter of the subjects had to be discarded.

B. The "normal range"

In the initial period of the research fifty children from the Orphanage and seventy-eight children from the Mission School in St. Anthony were tested. Both groups ranged in age from 6 to 17.

The Orphanage children received daily supplements of cod liver oil containing about 15,000 International Units (I.U.)[3] of vitamin A, or two and one-half times the highest estimates of optimal requirement. These children therefore represent a reliable normal control. The distribution of their visual thresholds is shown in figure 1. The mean log threshold is 2.17 and the spread about 1 logarithmic unit. Similar distributions of normal thresholds have been reported by Lindqvist ('38) and by Hecht and Mandelbaum ('39).

The Mission School children lived at home and ordinarily received no vitamin supplements. Nevertheless their threshold distribution, also shown in figure 1, was very similar to that of the Orphanage group. The mean log threshold in this instance was 2.25.

The impression is widespread that such a "normal range" of thresholds might provide the primary or even sole basis for the diagnosis of vitamin A deficiency. Actually it is

[3] Throughout this paper all vitamin A concentrations are expressed in the International Unit (I.U.), equivalent to one U.S.P. XI Unit, or to 0.6 μg. of pure β-carotene. Concentrations of thiamine (vitamin B_1) are expressed in International Units (U.S.P. XI), each of which is equivalent to the vitamin B_1 activity of 10 mg. of the International Standard adsorbate.

entirely inadequate for this purpose. High visual thresholds may result from a variety of congenital and acquired abnormalities other than deficiency in vitamin A. Conversely there is latitude enough for a threshold to rise considerably above its optimal value and still remain within the "normal range".[4]
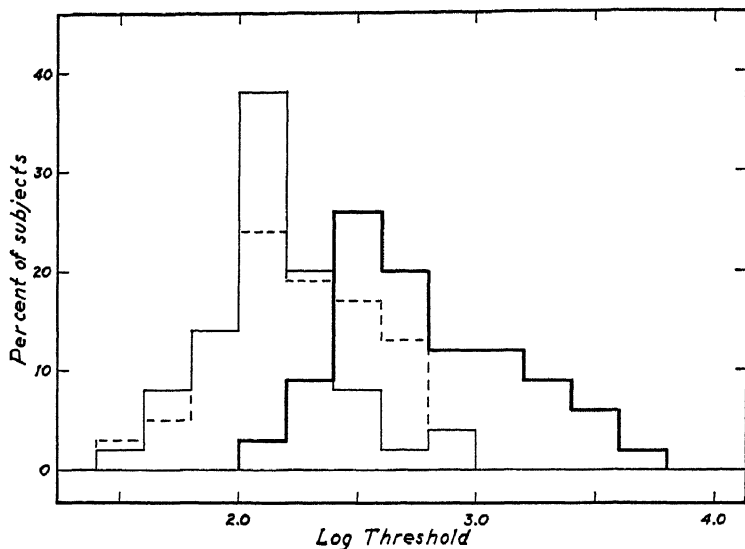
Fig. 1 Distribution of visual thresholds of fifty children optimally supplied with vitamin A (fine line); of seventy-eight unselected school children (broken line); and of thirty-four vitamin A deficient subjects (heavy line).

The threshold distribution of our vitamin A deficient subjects, as shown in figure 1, overlaps widely with the control distribution. About half of the vitamin A deficient cases possessed log thresholds well within the "normal range". Conversely, of those subjects who possessed log thresholds above 2.7, only half proved to be vitamin A deficient. Clearly

Case 1. Mrs. R., 38. Pregnant 8 months. Had had seal meat about twice weekly, brown flour, some butter; but no milk, fresh vegetables or game for months. Initial log threshold, 2.49. Three and one-half hours after administration of 100,000 I.U. of carotene her log threshold had fallen to 1.67. After 3 days supplementation with 10,000 I.U. of carotene the log threshold was virtually unchanged, 1.76. This patient's threshold had been displaced from the top to the bottom of the "normal range" by carotene administration.

a more searching criterion than low or high threshold is needed to estimate vitamin A deficiency.

C. The vitamin A-labile threshold

A subject may be regarded as vitamin A deficient when his visual threshold, whatever its initial level, is lowered significantly by administration of vitamin A, or is vitamin A-labile. We do not yet know that this definition is exhaustive, but it probably comes as close to the root of the matter as is at present possible.

Repeated measurements by our methods have shown that the threshold of a normal subject generally varies during one sitting within a range of about 0.1 log unit, and varies from day to day within a range of about 0.3 log unit, or about 100%. Only changes which transcend these limits, therefore, are significant in the present procedure.

Vitamin A-lability was determined as follows. The visual threshold of a subject having been measured, a known dose of a vitamin A preparation was given orally. Usually within several hours the threshold was redetermined. A decrease in threshold of at least 0.3 log unit, or eventual lowering by this amount over a longer period of supplementation, was accepted as evidence of vitamin A deficiency.

In general a threshold which reacted to a single dose of the vitamin did so within 1 to 6 hours. In some instances, however, significant changes were noted only after 24 hours to 48 hours, and additional supplementation. Usually if the first dose of vitamin A yielded no significant result, further doses were given and tests conducted over as long a period as the patient remained available. A subject whose threshold was not significantly lowered by at least 2 weeks of regular supplementation was regarded as non-deficient.

Originally vitamin A was given only to those subjects with log thresholds above 2.7. When this procedure had proved unreliable, the vitamin was given to all subjects with thresholds in the upper portion of the "normal range", and, re-

gardless of this, to all those with beriberi symptoms or poor
dietary histories. Ideally, the thresholds of all subjects should
have been tested for vitamin A-lability.

II. THE INCIDENCE AND DEGREE OF VITAMIN A DEFICIENCY

During the period of the survey, 353 persons were examined.
Their origins are shown in table 1, A. The fifty-eight Or-
phanage children form a distinct control group. Fifteen
patients hospitalized or receiving outpatient care for beriberi
constitute a group specially selected for nutritional disease.

TABLE 1

A. Distribution of persons tested

St. Anthony Orphanage	58
St. Anthony Mission School	82
Cartwright Mission School	51
St. Anthony Hospital and Outpatients	103
At home	59
Total	353

B. Vitamin A deficiency and beriberi

	CASES	AVERAGE AGES	MALES: FEMALES
Vitamin A deficiency alone	19	26.2	7:12
Beriberi alone[1]	17	39.6	13:4
Vitamin A deficiency and beriberi	15	41.1	13:2
Total vitamin A deficient	34	32.8	20:14
Total with beriberi	32	40.3	26:6

[1] This group includes a number of cases with thresholds in the "normal range"
but not completely tested for vitamin A-lability.

The remaining 280 subjects probably represent a fair sample
of the general population. They include eighty-eight patients
under hospital treatment for other causes than nutritional
deficiency; and seventeen subjects found at large with beriberi,
in apparent accord with the random distribution of such cases.

Vitamin A-labile thresholds were found in thirty-four sub-
jects, or 9.7% of the total number examined (table 1, B).
Of these, seven had been hospitalized for beriberi. The inci-
dence of vitamin A deficiency among the "random" subjects,
therefore, was 27 out of 280, or still 9.7%.

Most of these subjects exhibited low degrees of deficiency. About half of them possessed thresholds within the "normal range." At most nine subjects, or 3% of all those examined, might have been described as clinically night-blind. Only two complained of poor vision in dim light. No xerophthalmia or keratomalacia was encountered. We conclude that severe vitamin A deficiency rarely occurs in this population at present.

This situation is closely connected with the time required for night-blindness to develop. On experimentally A-deficient diets the visual threshold in some cases rises at once, in others only after several weeks or months (Wald and Steven, '39; Hecht and Mandelbaum, '40). The latter result appears at present to be the more usual. We have now encountered it frequently in the field. A considerable number of our subjects had subsisted for weeks or months exclusively on the regional staples, white bread, tea, and molasses or sugar. Almost all such persons displayed symptoms of beriberi, sometimes severe. Yet regularly they possessed visual thresholds well within the "normal range." [5] This prolonged resistance to vitamin A deficiency undoubtedly accounts in large part for the low incidence actually encountered.

About 5% of all the subjects examined possessed stable visual thresholds and good dietary histories and were almost certainly not vitamin A deficient, yet were highly night-blind.[6]

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[5] *Case 2.* E. P., female, 34. Beriberi following childbirth. Legs numb, no patellar reflex, anaesthetic to pin-prick. Unable to walk. Tightness in epigastric region, dyspnoea, edema before delivery. Had given birth to twins 44 days previously, of which one died on the way to the hospital, the other shortly after admission. Log threshold, 2.00.

[6] *Case 3.* E. M., 13, female. Cartwright Mission School. A normal, well-fed, healthy-looking girl. She had received daily supplements of cod liver oil since arriving at school more than 2 weeks before. Her log threshold averaged 3.80, or about 1 log unit above the upper limit of the "normal range". She was given about 50,000 units of vitamin A. After 2½ hours, her log threshold averaged 3.89. She was re-tested on the following day: log threshold, 3.71. The vitamin A had had no appreciable effect. This subject is markedly night-blind with no evidence whatever of vitamin A or other nutritional deficiency.

This proportion appears to be extraordinarily high, and may be due to the spread of a hereditary factor for night-blindness within this unusually inbred population.

III. VITAMIN A DEFICIENCY AND BERIBERI

Beriberi is endemic in Northern Newfoundland. The number of hospital admissions is only a crude index of its incidence. In general only severe cases are admitted, in which the limbs are paralyzed or the heart dilated. Symptoms of less severe deficiency are widespread among the population. A syndrome known locally as "Newfoundland stomach" is probably at least in part due to mild thiamine deficiency; its symptoms are prolonged constipation and a number of vague general complaints, such as irritability, dyspepsia, itching and burning sensations, and lassitude. Doctor La Salle of Port Saunders, Newfoundland, has treated this ailment with thiamine chloride and in some instances observed almost immediate relief of symptoms (personal communication).

In the succeeding discussion the term beriberi refers only to the acute disease, characterized by clinically clear neural or cardio-vascular disturbances. In all, thirty-two such cases, fifteen of which were sufficiently severe to require hospitalization, were tested for vitamin A deficiency. The results are listed in table 1, B.

Fifteen of the beriberi subjects possessed definitely vitamin A-labile thresholds. All the others possessed thresholds which were either stable to vitamin A, or well within the normal range. Some of the latter were not exhaustively tested for vitamin A-lability, and may possibly have included several incipient A-deficient individuals. In any event the incidence of vitamin A deficiency in the beriberi group is about five times as high as in the general population. This is not surprising, since the highly restricted diets common in this region are undoubtedly deficient in many factors simultaneously.

Beyond this, however, no intrinsic relation appears to exist between mild vitamin A deficiency and beriberi.[7] Several subjects with severe beriberi exhibited normal visual thresholds, stable to vitamin A (cf. case 2, above).[8] In patients with both deficiencies, administration of brewer's yeast alone did not lower the visual threshold significantly, while vitamin A alone was as effective as vitamin A and brewer's yeast together.[9]

Vitamin A usually lowered the thresholds of such subjects to well within the normal range. There is therefore no reason to believe that even a residue of night-blindness is due to beriberi itself.

IV. CORRELATIONS

Age and sex. (Table 1, B.) Both vitamin A and beriberi—excluding infantile beriberi with which we did not deal—are diseases of maturity in the region of this survey. Though more than half of all our subjects were school children, none

[7] *Case 4.* R. H. C., 54, male. Moderate symptoms of beriberi; log threshold, 3.6. Within 1½ hours after administration of 40,000 I.U. of carotene his threshold had fallen 0.75 log unit, or to about ⅙ its initial value. He was left some carotene and a small supply of yeast. Three weeks later he was admitted to hospital with more severe symptoms of beriberi. His visual threshold, however, had fallen still further, to 2.55. On regular daily administration of 25,000 units of vitamin A, the log threshold sank within 4 days to 1.9. It remained at this level until the patient's discharge 20 days later. This case demonstrates a very large improvement in visual threshold coincident with intensification of beriberi symptoms.

[8] *Case 5.* L. C., male, 28. Beriberi. Legs weak and at times edematous, hyperaesthetic to pin-prick. Tightness in epigastric region; dyspnoea. Could work only for short intervals. His diet throughout the winter had been white bread and tea; no meat or vegetables, negligible butter. Log threshold, 2.47. After taking 110,000 I.U. of vitamin A over a 3-day period, his log threshold was 2.30. This is not a significant change.

[9] *Case 6.* J. P., 41, male. Mild beriberi; initial log threshold, 2.95. Twenty-four hours following administration of brewer's yeast containing about 500 I.U. of thiamine the threshold had not significantly changed (2.8). One and one-half hours after administration of 50,000 I.U. of carotene the log threshold had fallen to 2.45, and on re-test 24 hours later had fallen to 2.25.

of the beriberi cases and only three of the vitamin A deficiencies were in the age range 6 to 15. Moreover, the vitamin A deficient subjects were in general much younger than those with beriberi. The average age of the former group was 26.2; or, with two exceptionally old male subjects excluded, 22.3. The average age of the beriberi cases was almost twice as great, 40.3.

These disorders also affect the sexes differentially. A small, perhaps not significant majority of the vitamin A deficient subjects were males. About 4/5 of the beriberi cases, however, were men; and half of the latter were simultaneously vitamin A deficient.

Season. Seasonal epidemics of night-blindness have been reported from the area of this survey (Appleton, '21; Aykroyd, '30). No epidemic was observed during the period of this research, nor did inquiry reveal any general incidence of night-blindness in this population in recent years. The visual thresholds of fourteen children at the St. Anthony Mission School were measured repeatedly during the entire course of the research, but they displayed no appreciable seasonal fluctuation.

Beriberi, however, attained a high peak in February, with seven cases, and in March, with sixteen. Nine of the March cases were simultaneously vitamin A deficient. These months, in which winter stores are running low and new supplies have not yet become available, represent the nutritional low of the year.

DISCUSSION

The response to vitamin A. The usefulness of our method for estimating vitamin A deficiency depends in part upon the speed with which the night-blind retina reacts to vitamin A. Some question has recently arisen concerning this matter. The following summary may clarify its present status.

1. Clinical studies of acute dietary night-blindness now embrace well over 1000 cases (Kubli, 1887, 320 cases; Birn-

bacher, '27, 330 cases; Bloch, '20–'21; Appleton, '21; Aykroyd, '30; Mori, '32). All these workers agree that nearly always the subjective and crude objective symptoms of night-blindness vanish within a few hours to a few days after administering a complete diet or vitamin A supplements. Judging by laboratory experience, the decreases in threshold which accompany such cures could not well be less than a full logarithmic unit.

2. Birnbacher's study included crude measurements of the visual threshold. He observed that though several meals containing roast mammalian liver almost invariably dissipated all clinical signs of night-blindness, in about half the cases the visual threshold continued to fall through a further period of supplementation. He rarely encountered patients in whom complete cures took longer than 4 weeks; though in a few extreme cases it occupied several months. It must be noted that by present standards Birnbacher's supplementation was very low. Our own observations are consistent with this report.

3. A few carefully controlled studies of experimental night-blindness have been recorded. Jeghers ('37 b), having produced mild night-blindness in himself, observed a significant improvement within 2 hours and complete cure within 3 days of heavy dosage with vitamin A. Wald et al. ('38, '39) repeatedly obtained complete reversals of mild night-blindness in two subjects within $1\frac{1}{2}$ to $2\frac{1}{2}$ hours after large oral doses of vitamin A or carotene. Lewis and Haig ('39) obtained similar results with three young children. Booher et al. ('39) found minimal doses of cod liver oil to take effect in five subjects within 2–3 hours after administration.

Hecht and Mandelbaum ('40), however, report that the complete reversal of night-blindness in all nine of their subjects required from 6 weeks to over 3 months of heavy vitamin A supplementation. Their results are unique; not because the observed responses were slow, but because they were invariably so. The consensus of all the clinical and most

other laboratory reports shows this slow type of response to be relatively rare.[10]

Hecht and Mandelbaum's charts as drawn suggest another peculiarity—occasional rise of visual threshold during heavy feeding with vitamin A concentrates. It may be hoped that these changes are not significant, since they fall within the authors' stated range of day-to-day variation.

4. Our criterion for vitamin A deficiency is a decrease in threshold of at least 0.3 log unit within at least 2 weeks of regular vitamin A supplementation. As an extreme test, this may be appraised in the light of Hecht and Mandelbaum's results. In six out of nine of their subjects tested with single large doses of vitamin A, the visual threshold decreased by 0.3 to 1.0 log unit within 3–8 hours. In two further subjects, the threshold fell by this amount within about a week of supplementation. The final subject required about 4 weeks for significant change. Another subject who had not been tested with single large doses responded still more slowly. In this extreme situation, therefore, our method, strictly applied, might have overlooked two out of these ten deficiencies.

From all the information now available it may be concluded that an undetermined but in any case small percentage of vitamin A deficient subjects might fail to respond within 2 weeks of regular supplementation. It has not yet been shown that this occurs to a significant degree under field conditions. Even this potential disability in the procedure, however, may be corrected by lengthening the period of supplementation in doubtful cases.

[10] Hecht and Mandelbaum cite in support of these observations the experiments of Steffens, Bair and Sheard ('39) and of McDonald and Adler ('40). The first reference is clearly in error; as the authors state, their three subjects failed to respond to vitamin A because they were not night blind. The second paper concerns a single subject whose threshold on an experimental diet rose about 1 log unit in 4 weeks, then took over 8 months of normal and supplemented diets to return to normal. It is extraordinary, not to obtain this result once, but to obtain it exclusively, as have Hecht and Mandelbaum. It is still entirely possible that this is a laboratory artefact, associated with certain conditions surrounding the production of experimental night-blindness.

Incidence of vitamin A deficiency. A number of recent researches purport to show that mild night-blindness is widespread within the general populations of European countries and the United States (cf. reviews by Jeghers, '37 a; Lindqvist, '38). These results were obtained with frankly empirical, arbitrary methods, the physiological meaning of which is obscure.

One procedure, developed by Jeans et al. ('36, '37) using the Birch-Hirschfeld Photometer or the Biophotometer, has revealed "abnormal" thresholds believed to be due to vitamin A deficiency in about 20–80% of various groups of American and English school children and adults. The Edmund test, based upon brightness discrimination and in wide use in the Scandinavian countries, has yielded at times even higher deficiency estimates. But Palmer and Blumberg ('37) and Isaacs, Jung and Ivy ('38) have failed to confirm these results with Jeans's methods and have seriously questioned their reliability. Lindqvist ('38) and Groth-Petersen ('38) have similarly criticised the Edmund procedure. Both methods as originally employed apparently suffered from an uncritical appraisal of the variability of the measurements, and of the limitations of a "normal range".

Another group of methods has employed the threshold of the dark adapted eye in some context which makes the procedure specific—for example, the determination of vitamin A lability or of blood vitamin A. Such methods have so far indicated a relatively low incidence of vitamin A deficiency. Lindqvist found abnormally high visual thresholds accompanied by abnormally low serum vitamin A concentrations in less than 5% of a group of hospital patients particularly suspected of vitamin A deficiency. Lewis and Haig ('40) found only one abnormally high and vitamin A-labile threshold among 144 children, mostly from poor homes, under hospital care in New York. The present survey, conducted upon a population chosen for its long history of deficiency diseases, reveals an incidence of vitamin A-labile thresholds of about 10%, more than half of them within the "normal range".

Probably more extensive use of controlled and accurate procedures will show that even very mild vitamin A deficiency ordinarily is rare in occidental populations.

SUMMARY

1. The incidence of vitamin A deficiency was surveyed by visual methods in an area from which epidemic night-blindness has been reported. With a specially designed portable adaptometer, the threshold of the completely dark adapted eye was measured. Rise of this threshold above a "normal range" proved inadequate as an index of deficiency. Deficiency is indicated reliably by a vitamin A-labile threshold—one that, whatever its initial level, is lowered at least 0.3 logarithmic unit within 2 weeks of regular vitamin A supplementation. In practice a threshold which reacted to vitamin A usually did so within 1-6 hours.

2. Vitamin A-labile thresholds were found in 9.7%, and clinical night-blindness in at most 3% of the subjects. About half the A-labile thresholds were within the "normal range". About half were found in subjects suffering also from beriberi.

3. Vitamin A-labile thresholds and beriberi are mutually independent. Acute beriberi may be accompanied by normal thresholds, stable to vitamin A. In subjects with both deficiencies, brewer's yeast does not lower the threshold, while vitamin A alone is as effective as vitamin A and brewer's yeast together.

4. Both vitamin A deficiency and beriberi are most prevalent in February and March. Both (infants excluded) are found

principally in adults. Vitamin A deficiency occurs in both sexes at average age about 26, beriberi primarily in males of average age about 40.

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DEPLETION OF TISSUE GLYCOGEN DURING FASTING AND FATIGUE AND PARTIAL RECOVERY WITHOUT FOOD

PAULINE E. NUTTER [1]

Department of Vital Economics, University of Rochester, New York

This study was made for the purpose of securing base-line values from which to calculate the recovery of glycogen at intervals of 1, 2 and 3 hours following fatigue and feeding with known amounts of glucose and fructose (see following paper: Nutter and Murlin, '41). A considerable amount of time also was spent in evaluation of the analytical methods for determination both of glucose and glycogen which, from the literature, appeared to be most appropriate to the purpose. Results of the methodological work will be described briefly first.

In control experiments with dry C. P. glucose the average recovery was 99.67% by the copper-iodometric method of Shaffer and Somogyi ('33); 100.54% by direct iodometric oxidation as described by Bierry, Gouzon and Magnan ('36); and 104.57% by Bierry and Goiran's ('28) micro modification of Bertrand's ('06) cupric procedure. With the last-named method great difficulty was experienced in reaching an accurate end point, and wide variations in results were obtained. Since the later method of Bierry et al. ('36) offered no advantage, the Shaffer-Somogyi method was adopted.

The effect of various factors upon glycogen recovery was investigated thoroughly, and as a result the method of Good, Kramer and Somogyi ('33) was adopted after modification

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as follows: Tissues were dissolved in 45 instead of 30% KOH, glycogen was precipitated by addition of 3 instead of 1.1 volumes of 95% alcohol, and this was followed by a second precipitation in the same manner. Recovery of added dry "C. P." glycogen[2] on the basis of the actual glycogen was 99.19%.

PROCEDURE

Young adult albino rats, falling within a restricted range of weight (averaging about 140 to 150 gm.), and nearly all of the female sex, were selected from the stock colony. They had been maintained previously on a uniform diet.[3] Food was removed from the cages 24 hours prior to the beginning of the experimental run, but the animals were allowed drinking water ad libitum. To avoid, so far as possible, the variations due to diurnal changes in glycogen storage observed by Forsgren ('28) as well as by Ågren, Wilander and Jorpes ('31), the experiments were arranged so that the tissue samples would be taken at a fairly uniform time, namely, late in the afternoon.

Attempts to fatigue the rats by voluntary exercise in revolving cages were not successful, due to wide individual variations in inclination for this kind of work. Neither the sight of food nor exposure to cold or darkness, or both, stimulated any greater activity. Later trials with a treadmill were also unsatisfactory, since, with a few exceptions, even repeated electrical stimuli were ineffective in forcing the animals to run. In the experiments herein reported, the rats, fasted for 24 hours prior to the experiment, were fatigued by swimming in water at 30°C. until exhausted[4] (a period of some 8 to 11 hours), after which they were dried and placed in warm cages.

[2] Pfanstiehl.

[3] Purina Dog Chow.

[4] Non-fatigued animals, when removed from the swimming tank, run about the floor in normal fashion. The exhausted animals show little inclination to move and apparently lack powers of coordination so that they stagger about in a drunken manner.

In order to establish a basal level of glycogen, fasted and fatigued controls were allowed to recover, without carbohydrate or other food, for 1, 2 and 3 hours. In preliminary experiments the animals were prepared for the removal of tissue samples by stunning them with a blow on the head. This method was abandoned because of the frequently accompanying convulsive movements, with consequent glycogenolysis in the muscles. Careful administration of sodium amytal obviated any struggling in the animal.

Therefore, at the end of the recovery period, the animals were anaesthetized by intraperitoneal injection of 10% sodium amytal, prepared according to the method of Mulinos ('28), the dosage being 10 mg. per 100 gm. of female rat and 20 mg. per 100 gm. of male rat (Nicholas and Barron, '32). After anaesthesia was established, the abdomen was opened and a lobe of liver tissue was rapidly excised. The blood was soaked up in filter paper and the sample was immediately dropped into a tared tube of warm 45% KOH solution. The gastrocnemius muscle was then extirpated rapidly, with precautions to avoid twitching or damaging of the fibers, and was also dropped into KOH. The vastus lateralis muscle was used in a very few of the earlier experiments, but it was found that the gastrocnemius could be dissected out more quickly and without cutting into the muscle fibers. After determination of their weights, the tissue samples were dissolved by digestion for about ½ hour in a boiling water bath with frequent shaking to hasten the disintegration.

EXPERIMENTAL RESULTS

According to the usual custom, all tissue glycogen values are calculated in terms of milligrams of glucose, but expressed as glycogen. Table 1, containing representative examples of the values obtained for the glycogen content of paired muscles, shows that large differences (0.36% to 38.45%) occurred after stunning the animal, whereas closely agreeing results (varying from 0.06% to 0.67%) were obtained under amytal anaesthesia. It is interesting to note that, in the animals

TABLE 1

Glycogen content of opposite paired muscles after stunning and under amytal anaesthesia

RAT	BODY WEIGHT	METHOD OF TREATMENT	GLYCOGEN		DIFFER-ENCE	DIFFER-ENCE
			In right muscle	In left muscle		
	gm.		mg.%	mg.%		%
7	220	Killed by stunning	409.4	316.8	92.6	22.62
18	115	Killed by stunning	208.6	205.4	3.2	1.53
20	135	Killed by stunning	141.0	141.1	0.1	0.71
27	150	Killed by stunning	375.3	346.5	28.8	7.67
38	149	Killed by stunning	332.5	329.5	3.0	0.90
37	135	Killed by stunning	304.7	254.2	50.5	16.57
9	185	Killed by stunning	174.5	107.4	67.1	38.45
51	197	Killed by stunning	333.1	331.9	1.2	0.36
56	182	Anaesthetized by amytal	349.6	349.3	0.3	0.09
58	215	Anaesthetized by amytal	605.8	609.9	4.1	0.67
69	206	Anaesthetized by amytal	348.1	347.9	0.2	0.06
93	144	Anaesthetized by amytal	164.2	164.6	0.4	0.24
116	143	Anaesthetized by amytal	327.7	329.9	2.2	0.67
128	119	Anaesthetized by amytal	516.2	518.8	2.6	0.50
78	160	Anaesthetized by amytal	104.2	104.3	0.1	0.10

killed by stunning, there is frequently a decidedly lower glycogen content in the muscle of the left leg which was last to be sampled, indicating that glycogenolysis occurs very rapidly in the dead organism.

Table 2 summarizes the results of different procedures in depleting the stores of muscle glycogen. The effects of fasting different lengths of time, of different types of work, of cold and of different periods of swimming are perfectly evident from the table. Thirty degrees Centigrade was considered to be about the optimum temperature of the water for swimming rats. Water at 25°C. produced fatigue and depletion of the glycogen stores much more quickly, but the animals reached the point of exhaustion so suddenly, or became so chilled and stiff, that they would abruptly quit swimming and drown.

Table 3 summarizes the average findings for liver and muscle glycogen content in the control rats: (a) normal, fed animals taken directly from the stock colony; (b) 24-hour fasted animals; (c) rats fasted for 24 hours and exercised

TABLE 2
Effect of fasting, cold and exercise on muscle glycogen

CONDITION OF RATS	NO. OF RATS	AV. WT. OF RATS	TYPE OF EXERCISE	AMOUNT OF EXERCISE	HOURS IN COLD	GLYCOGEN AS MG. OF GLUCOSE PER 100 GM. MUSCLE
		gm.				
Normal, fed	16	166	0	0	0	571.8
24-hour fasted	5	129	0	0	0	379.7
45-hour fasted	6	152	0	0	0	304.2
45-hour fasted	5	142	0	0	6	235.2
45-hour fasted	10	154	Running in revolving cage	1284 revolutions	6	300.6
81-hour fasted	4	105	Running in revolving cage	8917 revolutions	0	172.7
24-hour fasted	4	165	Running on treadmill	4 hours	0	308.5
24-hour fasted	3	185	Swimming	30 minutes	0	286.5
24-hour fasted	6	142	Swimming	1½ hours	0	193.2
24-hour fasted	7	128	Swimming	5 hours	0	93.3

TABLE 3

Glycogen content of control rats

CONDITION OF RATS	NUMBER OF RATS		AVERAGE WEIGHT OF RATS	MG. OF GLYCOGEN PER 100 GM. OF LIVER	MG. OF GLYCOGEN PER 100 GM. OF MUSCLE	CALCU- LATED TOTAL LIVER GLYCOGEN	CALCU- LATED TOTAL MUSCLE GLYCOGEN
	Liver	Muscle					
			gm.			*mg.*	*mg.*
(a) Normal, fed	4♀	13♀ 3♂	166	2778.6	571.8	138.4	474.6
(b) 24-hour fasted	3♀	5♀	132	33.8	379.7	1.3	244.9
(c) 24-hour fasted fatigued	19♀	19♀	144	21.5	70.9	0.9	51.0
(d) Fasted, fatigued, 1-hour recovery	19♀	19♀	147	25.4	101.0	1.1	74.2
(e) Fasted, fatigued, 2-hour recovery	16♀	16♀	144	23.5	119.8	1.0	86.3
(f) Fasted, fatigued, 3-hour recovery	15♀	15♀	154	69.4	121.6	3.2	93.6

by swimming until fatigued; (d), (e), and (f) rats fasted for 24 hours, fatigued by swimming, and allowed to recover for 1, 2 and 3 hours respectively. The values are expressed as milligrams per 100 gm. of tissue, and also as calculated total amounts, assuming (Cori, '31) that the muscles constitute about 50 and the liver only 3% of the body weight. Sixteen normal, fed rats showed a liver glycogen content of 2779.0 mg. per cent and a muscle glycogen of 572 mg. per cent. After 24 hours fasting the liver glycogen was reduced by one-third during this period of starvation. Fatiguing exercise decreased the liver glycogen only slightly, but brought the muscle glycogen down to a very low level, i.e., 71 mg. per cent, or about one-eighth of the amount in normal, fed animals. During the recovery period following fatigue, a small amount of glycogenesis occurs. The liver glycogen is not much affected until the third hour, when it reaches a level higher than that observed after fasting only. The muscle glycogen rises steadily during the first 2 hours, with little change in the third hour. These control values observed in the fatigued rats after 1, 2 and 3 hours recovery without food were taken as the base line in determining "new glycogen" formation, or the increase above the pre-formed level, in the sugar fed rats (see following paper).

DISCUSSION OF RESULTS

As a primary requisite in glycogen studies, the experimental animals should be of identical sex, of limited age range and previously maintained on a uniform diet. Stöhr ('32) noted a higher level of liver and muscle glycogen in male than in female rats after a 24–32-hour fast. The liver glycogen of the male rat diminished with increasing age, whereas the old females showed a marked increase in liver glycogen. Deuel, Gulick, Grunewald and Cutler ('34) confirmed the existence of significant sexual variations in carbohydrate metabolism. These authors found liver glycogen to be higher in fasting male than in female rats 24, 48 and 72 hours after glucose administration. There was no difference between the sexes

killed without fasting after a high carbohydrate diet. Muscle glycogen was not appreciably different in the two sexes.

The composition of the diet on which experimental animals have been previously maintained is a factor of considerable importance in establishing a base-line glycogen level (Greisheimer and Johnson, '30; Sahyun, Simmonds and Working, '34; MacKay and Bergman, '33). After having been weaned the rats used in this investigation were fed uniformly on the commercial chow and nothing else.

The time of fasting in the present work was limited to 24 hours, since this period certainly brings the animal into a postabsorptive condition and produces a sufficiently marked depletion of the glycogen stores. Longer fasting appears to be contraindicated, since the decrease in glycogen after 24 hours is very slow (22 mg. between the twenty-fourth and forty-eighth hour, according to Cori and Cori, '28) and since prolonged starvation diminishes the rate of absorption and utilization of carbohydrates (Cori, '27–'28; MacKay and Bergman, '33; Goldblatt, '25).

Since Page ('23) introduced amytal as an anaesthetic which is without effect on the blood sugar level of dogs or rabbits, it has been used with approval by many investigators. In the experience of this laboratory dogs in long experiments under amytal do show significant changes in blood sugar (Driver and Murlin, '41), but neither this type of effect, the interference with absorption reported by Olmsted and Giragossintz ('29), nor the inhibition of glycogen recovery noted by Long ('28) could come into consideration in the use of amytal as a means of securing tissue only.

In this investigation, the tissue samples were not frozen, contrary to the recommendation of numerous authors. It has been observed that freezing causes well-defined tetanic contractions of muscle, which Steiner ('35) found to result in a small loss of glycogen, reflected in a rise in both fermentable sugar and hexosemonophosphate.

After exhausting exercise the depleted glycogen reserves of liver and muscle are gradually replaced, even in the

absence of food. The processes involved are probably the
same ones which are responsible for the increased storage
observed during the nocturnal portion of the diurnal cycle,
and may include, to some extent, migration of glycogen from
the reservoirs in the skin and the bony and cartilaginous parts
of the body.

Since Evans, Tsai and Young ('31) were unable to account
for the combustion and liver glycogen deposition occurring
in fasting cats recovering from experimental glycogenolysis,
on the basis of observed changes in blood glucose, blood
lactate and muscle glycogen, they concluded that the restora-
tion must be at least partly due to processes of gluconeo-
genesis. Brand and Krogh ('35) also attributed the endoge-
nous increase in liver and muscle, during recovery from
exhausting work, to a small new formation of carbohydrate
from non-carbohydrate sources. Although experimental proof
was lacking, protein seemed a more probable source than fat.

The observation of Ågren et al. ('31) that the diurnal vari-
ation in liver glycogen is accompanied by a parallel variation
in nitrogen output (i.e., increased deamination with increased
glycogen deposition) indicates that glyconeogenesis may occur
partly at the expense of body proteins during recovery from
fatigue as well as during the normal diurnal cycle. The glycerol
fraction of catabolized body fat possibly contributes its share.
It is very likely also that lactic acid, accumulating in the
tissues and blood as a result of the exercise, is a potential
source of this "new glycogen." Its role, however, must be
relatively unimportant in view of the work of Long and
Grant ('30) showing that in the 24-hour fasted rat, where
the carbohydrate stores are extremely low, the resynthesis of
muscle glycogen after exercise is very slow, as compared with
the rate at which lactic acid is removed from the blood.

No attempt was made in this investigation to prove that
the recovery took place by glyconeogenesis either from fat or
from protein. By analogy with previous work already cited,
it is reasonably certain, however, that newly-formed carbo-
hydrate must have contributed a share. The transfer of

glycogen from other tissues, such as the skin, should be controlled to make this conclusion final.

The values obtained in the tissue analyses of the control animals compare very favorably with the figures to be found in the literature. The liver glycogen content of 2.78%, found in the unfasted rats, is somewhat lower than the level of 3.53% reported by Deuel, Gulick, Grunewald and Cutler ('34), but can probably be explained on the basis of the carbohydrate level of the previous diet, and the time of day at which the samples were taken. The muscle glycogen content of 572 mg. per cent, found in the unfasted animals, is a normal figure. The liver glycogen value after 24 hours without food, as reported in the literature, varies from 0.06 to 0.70% for female rats. Long and Grant ('30) found a value of 0.08%. The slightly lower value found in this investigation, 0.03%, can probably be explained as above. The muscle glycogen content of 380 mg. per cent, found after a 24-hour fast, is almost identical with the figure of 383 mg. per cent reported by Deuel et al. ('34). Long and Grant's ('30) observation that "the changes in liver glycogen during and after exercise are so small that they are insignificant" is amply confirmed by the data of table 3. This work also bears out the finding of earlier investigators, who noted a marked decrease in muscle glycogen during exercise and a gradual reformation during recovery without food.

SUMMARY

The glycogen content of paired muscles removed from rats killed by a blow on the head was compared with that of animals anaesthetized with amytal. Much more closely agreeing results were obtained from the live anaesthetized animals.

The effect of fasting, of exposure to cold, and of various kinds and amounts of exercise (running ad libitum in revolving cages, forced running on a treadmill, and swimming in water at different temperatures) was investigated. A 24-hour fasting period reduced the liver glycogen to a very low

level, which was depressed only slightly more by the fatiguing exercise. It reduced the muscle glycogen by one-third; after 24 hours the rate of fall was much slower. Fatiguing exercise while fasting decreased the muscle glycogen content to a much lower level. During the recovery period after fatigue by swimming 8 hours, a small amount of glycogenesis occurred, notwithstanding the fasting state. The liver glycogen was not much affected until the third hour, when it reached a level twice as high as that observed after fasting only. The muscle glycogen rose notably during the first 2 hours, but only slightly in the third hour.

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GLYCOGEN FORMATION IN LIVER AND MUSCLE FROM GLUCOSE AND FRUCTOSE AFTER EXTREME MUSCULAR EXHAUSTION [*]

PAULINE E. NUTTER [1] AND J. R. MURLIN

Department of Vital Economics, University of Rochester, New York

THREE FIGURES

The two principal monosaccharides occurring as such or in combination in human foods differ sufficiently in their chemical and physical properties to produce plainly measurable differences in their absorption rates, in their rates of combustion and their specific dynamic effects. How much under extreme need would they differ in restoration of glycogen to the tissues suffering most from muscular exhaustion? The previous study (Nutter, '41) showed that swimming was the best of several methods tried for producing an extreme reduction in the glycogen of muscles as well as of liver. Also that a slight amount of restoration of glycogen in both tissues occurs following extreme exhaustion without feeding. It turned out that this spontaneous recovery of glycogen without visible supply of carbohydrate was quite small and might have been neglected so far as the comparative effects of fed glucose and fructose were concerned. What could not be neglected, however, was the difference in rate of absorption of the two hexoses. An "index of glycogen" formation was therefore devised which takes into account both absorption rates and those of glycogenesis.

PROCEDURE

The experimental animals were fasted and fatigued by swimming exactly as the controls (Nutter, '41). After removal from the water, and drying, they were fed by stomach tube 2.5 cc. of the sugar solutions to be tested. All solutions were brought to 40°C. before feeding. In the earlier experiments, in which absorption was allowed to proceed for 2 hours, the fed sugars were 50% glucose [2] and 50% fructose.[3] The fructose feeding frequently resulted in diarrhea, and animals thus affected were discarded. In the next series, in which absorption proceeded for 3 hours, diarrhea invariably followed the feeding of 50% fructose. Consequently, the concentration of both sugars was reduced to 40%. Slight symptoms often appeared even then after fructose, and in such cases the excreta were collected and added to the unabsorbed residues from the gastrointestinal tract. At the end of the 1-, 2- or 3-hour absorption period the sugar-fed rats were anaesthetized and samples of muscle and liver were obtained. Especially after the 1-hour absorption period, the stomach was still so full that regurgitation often occurred during the operative manipulations. To prevent loss of solution through the mouth, the abdomen was opened as the first step, and the esophagus was closed off with an artery clamp, before the tissue samples were excised.

Absorption was determined in the usual way by the so-called [4] Cori ('25) method with all precautions taken to prevent possible losses.

The liver and muscle glycogen content was determined by the method previously described, final estimation of the sugar produced by acid hydrolysis of the glycogen and all other sugar determinations being made by the copper-iodometric

[2] Supplied by the Corn Industries Research Foundation.

[3] Pfanstiehl, C. P.

[4] This method of killing animals of similar weight at different intervals after feeding a uniform amount of a food and analyzing the contents of the alimentary tract in order to determine the progress of digestion and absorption was originally introduced by Schmid-Muhlheim (1879) and was used very successfully by Kugler ('19) for absorption of protein.

titration method of Shaffer and Somogyi ('33), with corrections for non-fermentable reducing substances where necessary.

EXPERIMENTAL RESULTS

As previously described, in the 2-hour experiments the sugars were fed in two different concentrations, first as 50% solutions, and later as equal volumes of 40% solutions. As shown in table 1, a decrease in the concentration of sugar fed, either glucose or fructose, is accompanied by a decrease in the total amount of sugar absorbed and also by a decrease in the amount of sugar absorbed per 100 gm. of body weight.

TABLE 1

Effect of concentration on sugar absorption (2-hour absorption period)

SUGAR	CONCEN-TRATION	AMOUNT FED	TOTAL AMOUNT SUGAR ABSORBED	SUGAR ABSORBED IN MILLIGRAMS 100 GM. BODY WEIGHT
	%	mg.	mg.	
Glucose	50	1014.6	580.2	442.7
Glucose	40	761.5	340.5	233.4
Fructose	50	1160.7	504.4	329.5
Fructose	40	791.8	287.7	202.2

It should be noted (table 2) that glucose is always absorbed at a more rapid rate than is fructose, and also that there is a falling off in the rate as absorption proceeds. When the absorption rate is plotted against time, including all experiments and all concentrations of the sugar solutions fed, the decrease in rate is observed in the third hour. When the absorption rate is plotted against time, including only those experiments in which approximately the same concentrations of sugar were fed, the decrease in rate occurs in the second hour, giving S-shaped curves (fig. 1).

Table 2 summarizes the findings for new glycogen formation in the liver, and presents the corresponding data for muscle, the values being expressed as milligrams of new glycogen per 100 gm. of tissue (actually calculated as milligrams of glucose). The results show that glucose exceeds

fructose somewhat in restoring muscle glycogen during the 3 hours of observation and also liver glycogen for the first 2 hours. During the third hour considerably more glycogen is deposited in the liver from fructose, than from the aldo-hexose. In view of the faster rate of absorption of glucose it was necessary, in order to compare the new glycogen forma-
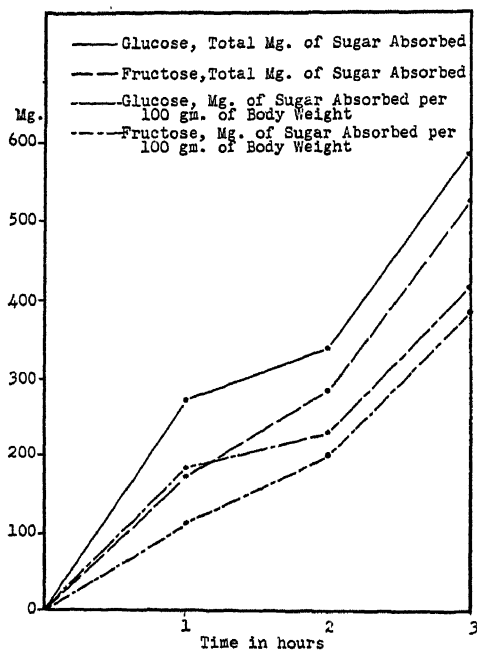
Fig. 1 Absorption of glucose and of fructose in 1-, 2- and 3-hour periods after feeding to exhausted rats.

tion from the hexoses on the basis of equal amounts of ab-sorbed sugar, to establish an "index of glycogen formation," i.e., the ratio:

$$\frac{\text{new glycogen (expressed as milligrams glucose per 100 gm. of tissue)}}{\text{milligrams sugar absorbed per 100 gm. of body wt.}}$$

In the liver (table 2, fig. 2) the glycogen index for glucose is ten times that for fructose at the end of 1 hour. By the close of the second hour, fructose has nearly equalled glucose

TABLE 2

Glycogen formation in the recovery process after fatigue, "index of glycogen" formation in liver and leg muscles

CONDITION OF RATS	NO. OF RATS	AV. WT. OF RATS	MG. OF SUGAR FED	MG. OF SUGAR ABSORBED PER 100 GM. BODY WT.	MG. TOTAL GLYCOGEN PER 100 GM. TISSUE	MG. NEW GLYCOGEN PER 100 GM. TISSUE	INDEX OF GLYCOGEN FORMATION IN TISSUE	STANDARD DEVIATION, GLYCOGEN INDEX	STANDARD ERROR OF THE MEAN
		gm.							
Liver									
Fasted, fatigued k. 1 hour after glucose	11♀	146	787.1	187.4	127.5	102.1	0.516	0.596	±0.180
Fasted, fatigued k. 2 hours after glucose	7♀	148	761.5	233.4	228.4	204.9	0.806	0.725	±0.274
Fasted, fatigued k. 3 hours after glucose	2♂ 12♀	142	825.8	415.2	804.5	735.1	1.689	1.216	±0.325
Fasted, fatigued k. 1 hour after fructose	13♀	154	792.0	115.1(61)	30.7	5.3	0.052	0.276	±0.077
Fasted, fatigued k. 2 hours after fructose	7♀	139	791.8	202.2(87)	185.3	161.8	0.750	0.880	±0.333
Fasted, fatigued k. 3 hours after fructose	1♂ 13♀	136	824.9	389.7(94)	1855.9	1786.5	4.605	1.678	±0.448
Leg muscle									
Fasted, fatigued k. 1 hour after glucose	11♀	146	787.1	187.4	165.0	64.0	0.327	0.379	±0.114
Fasted, fatigued k. 2 hours after glucose	3♂ 13♀	139	903.9	351.1	377.3	257.5	0.661	0.394	±0.099
Fasted, fatigued k. 3 hours after glucose	2♂ 13♀	142	825.8	415.2	415.6	294.0	0.687	0.307	±0.079
Fasted, fatigued k. 1 hour after fructose	13♀	154	792.0	115.1(61)	118.3	17.3	0.111	0.637	±0.177
Fasted, fatigued k. 2 hours after fructose	2♂ 15♀	147	1008.8	277.1(79)	255.2	135.4	0.459	0.340	±0.082
Fasted, fatigued k. 3 hours after fructose	1♂ 13♀	136	824.9	389.7(94)	367.8	246.2	0.644	0.237	±0.063

k. = killed.
Figures in ( ) = percentage rates as compared with glucose.

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in glycogenic power in the liver, and at the end of the third hour, fructose shows a glycogen index over two and one-half times that for glucose. In the muscle the differences are not so remarkable. Although the glycogen index for glucose is almost three times that for fructose at the end of the first
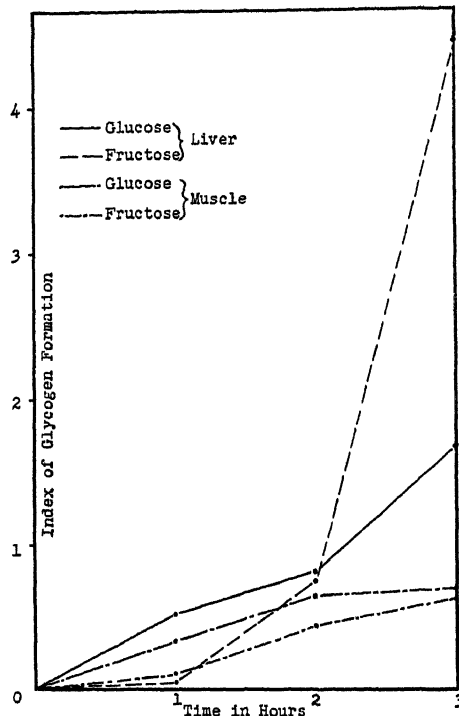
Fig. 2   Glycogen formation at intervals after feeding glucose and fructose, immediately following exhaustive fatigue, expressed as "glycogen index" (see text).

hour, after 2 hours it is less than one and one-half times as great, and by the end of the third hour, fructose shows almost as great a glycogenic capacity as glucose.

Assuming that the muscle tissue accounts for 50, and the liver for 3% of the body weight (Cori, '31), the total amount of new glycogen formed in liver and muscle from glucose and fructose was estimated, and the percentage of the ab-

sorbed sugar which was deposited as liver and as muscle glycogen was calculated. In total amount of new glycogen formation (these results are omitted in the interest of brevity) fructose falls somewhat behind glucose during the first 2 hours in the liver, but forges far ahead in the third hour. In the muscle, fructose lags behind during each of the 3 hours.
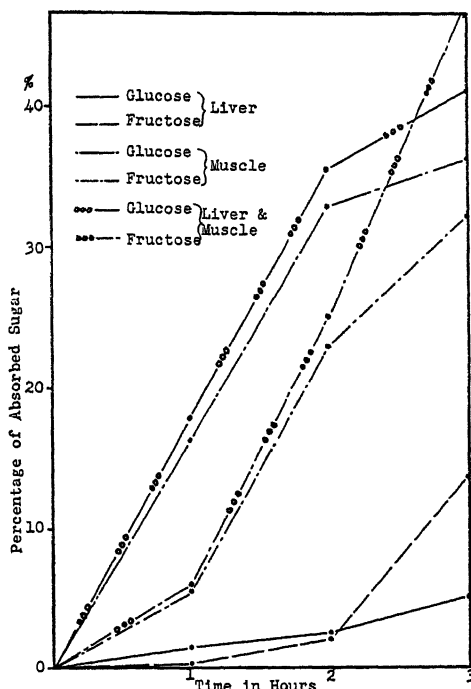
Fig. 3 Glycogen formation at intervals after feeding glucose and fructose, immediately after exhaustive fatigue, expressed as percentage of absorbed sugar laid down in liver and muscle and in both together.

When the percentage of absorbed sugar that is laid down as liver and muscle glycogen is calculated, glucose is found to be nearly ten times as effective as fructose in forming liver glycogen during the first hour (fig. 3). In the second hour fructose is almost as active as glucose, and by the end of the third hour the percentage of absorbed fructose deposited

in the liver is over two and one-half times the value for glucose. In the muscle the activity of the absorbed fructose in building up glycogen is less than half that of glucose in the first hour. In the second hour, fructose still lags behind, while in the third hour the keto sugar closely approaches glucose. Plotting the total percentage of absorbed sugar deposited in liver and muscle against the time in hours, one observes (fig. 3) that the rate is constant for glucose during the first 2 hours, falling off in the third hour. In the case of fructose, there is a lag in the first hour, followed during the last 2 hours by a constant rate which, curiously, very nearly parallels the initial rate for glucose.

Comparison of the glycogenic powers of the two hexoses on the basis of equal amounts of sugar absorbed, by means of the index of glycogen formation, or comparison of the percentage utilization of the absorbed sugars in total formation of liver and muscle glycogen, indicates that glucose is superior to fructose as a glycogenic agent in the early stages of recovery from fatigue, even when allowance is made for the difference in rates of absorption. However, as the time of recovery is prolonged, i.e., to 3 hours, the difference between the glycogenic capacities of the two sugars decreases in muscle, and in the liver fructose is stored to a much greater extent than is glucose.

DISCUSSION OF RESULTS

Cori's ('25) conclusion that the rate of absorption of the hexoses is independent of the absolute amount and concentration of sugar present in the intestine, giving a straight-line relationship between absorption rate and time, is definitely not confirmed by the observations on fatigued rats herein reported (table 1). Cori also concluded from data based on hourly determinations of the absorption coefficient (absorption per 100 gm. of body weight per hour), that fructose is absorbed at only 0.43 the rate observed for glucose. Burget, Moore and Lloyd's ('32) observation that glucose is absorbed only 9% more rapidly than fructose was made on a much

larger and better controlled series of animals. In the present investigation using fatigued rats, in which the absorption coefficients varied widely, the average results showed fructose to be absorbed at 61% of the rate for glucose during the first hour, 79 to 87% during 2 hours, and 94% during 3 hours (table 2). Factors responsible for the rather wide deviations observed in the amount of sugar absorbed during any given time interval may include: irregularities in the emptying rate of the stomach, variations in the concentration and pressure of the solution in contact with the intestinal mucosa due to secretion of variable amounts of water into the intestinal lumen, and varying degrees of disturbance of gastrointestinal motility due to the psychic effects of the exercise.

The data also contradict the conclusion of Cori ('25) and Cori, Cori and Goltz ('29) that the absorption rate of the hexoses is constant. The falling off in rate as absorption proceeds, as observed in the present investigation, cannot be due to lack of sufficient sugar to maintain the initial rate, for at the end of the third hour the gastrointestinal tract still contained more sugar than had been absorbed during the first hour. The significance of Cori's conclusions diminishes considerably when one considers that they are based on observations made on groups of rats each containing an average of less than five animals, and that his recorded data show a range of variation between the maximum and minimum ''absorption coefficients'' which in some groups reaches or exceeds 50% of the average value. Obviously the average results from such small groups, in which so wide a range of variation occurs, cannot be considered very significant. Similar investigations in our own and in other laboratories show these wide animal variations, indicating that long series of experiments are necessary before any valid conclusions can be drawn from the average results. The S-shape of the absorption rate curve obtained by plotting only those experiments in which approximately equal amounts of sugar were fed corresponds well with the results reported by Feyder and Pierce ('35) for average total absorption of glucose and sucrose by unfatigued

rats during 1-, 2- and 3-hour periods. Any explanation of the S-shape of the curve must of necessity be rather hypothetical. However, the following mechanisms might be suggested. In the initial stages absorption proceeds very rapidly, due to the high concentration of the sugar solution fed. This concentrated solution is extremely irritating to the gastrointestinal mucosa, and stimulates the secretion (aided by osmotic pressure) of large quantities of water into the intestinal lumen.[5] Due to the diluting effect of the alimentary secretions, absorption of sugar proceeds more slowly during the second hour. But the water is reabsorbed at a rate faster than the sugar passes through the intestinal wall, so that by the third hour the solution is again fairly concentrated, and absorption of sugar proceeds at a faster rate. Other factors besides concentration are probably involved, including pressure effects and the emptying rate of the stomach.

In order to determine the significance of the differences in the effectiveness of glucose and fructose in forming new liver and muscle glycogen, the data were subjected to statistical analysis (Davenport and Ekas, '36). The actual difference between the means was compared with the square root of the sum of the squares of the two standard errors of the means, expressed by the formula:

$$\sqrt{SE_{M_1}{}^2 + SE_{M_2}{}^2}$$

A significant difference between two means is one which exceeds twice this root, i.e.,

$$\frac{M_1 - M_2}{\sqrt{SE_{M_1}{}^2 + SE_{M_2}{}^2}} > 2$$

This ratio will be referred to as the significance ratio.

[5] That this process does occur is a proved fact. One hour after feeding these strong solutions, the stomach and upper intestine are found to be greatly distended. Within the following hour much of the fluid is reabsorbed. Abbott, Karr and Miller ('36) found glucose concentrations in the jejunum and ileum of human subjects ranging from 0 to 5%, regardless of the volume and concentration of solution ingested. The data showed that a very rapid inflow of fluid occurred from the walls of the stomach and duodenum.

Calculations thus performed (table 3) show that in the liver the superiority of glucose over fructose in restoring glycogen, during the first hour of recovery in the fatigued rat, is statistically significant, the actual difference being considerable, and the significance ratio being 2.4. By the close of the second hour fructose has nearly equalled glucose in glycogenic power in the liver, since the actual difference in the means (mean index of glycogen formation and mean percentage utilization) is very small and the significance ratio of the difference in the means is only 0.13. The superiority of fructose at the end of the third hour is very significant, with a ratio of 5.3. Although the average results for new glycogen formation in muscle indicate a greater effectiveness for glucose, especially in the first hour, the difference between the two sugars is not significant at any time. That the value of the significance ratio falls below 2.0 for the two sugars in building up muscle glycogen is partly to be accounted for, at least in the first 2 hours, by the extremely wide variations observed in the individual experiments. It is very likely that extension of the series to include a much larger number of animals might prove glucose to be significantly superior to fructose as a muscle glycogen former in the early stages of recovery after fatigue. The experimental groups reported here are relatively large, each containing eleven to nineteen animals, with the exception of 2-hour liver determinations on seven animals for each sugar.

Several causative factors may be suggested in explaining the initial slow rate of glycogen production. Perhaps compounds more fundamental than glycogen to the organism must first be replenished. Depression of insulin production during the previous fasting period is probably involved. In the early stages of the recovery process a high rate of carbohydrate combustion is quite conceivable. As a result of the prolonged period of strenuous exercise, there probably exists a decided condition of acidosis, due to accumulation of lactic acid and acetone bodies. Thus it seems reasonable to expect that a considerable carbohydrate combustion would be necessary

TABLE 3

Statistical comparison of glycogen formation from glucose and fructose

	NO. OF EXPTS.	MEAN	STANDARD DEVIATION	STANDARD ERROR OF THE MEAN	$\sqrt{S.E._{M_1}{}^2 + S.E._{M_2}{}^2}$	$M_1 - M_2$	SIGNIFICANCE RATIO $\dfrac{M_1 - M_2}{\sqrt{S.E._{M_1}{}^2 + S.E._{M_2}{}^2}}$
Index of glycogen formation in liver							
1-hour glucose	11	0.516	0.596	±0.180	0.196	0.464	2.367
1-hour fructose	13	0.052	0.276	±0.077			
2-hour glucose	7	0.806	0.725	±0.274	0.431	0.056	0.130
2-hour fructose	7	0.750	0.880	±0.333			
3-hour fructose	14	4.605	1.678	±0.448	0.553	2.916	5.273
3-hour glucose	14	1.689	1.216	±0.325			
Per cent of absorbed sugar deposited as liver glycogen							
1-hour glucose	11	1.54	1.80	±0.54	0.58	1.38	2.379
1-hour fructose	13	0.16	0.81	±0.22			
2-hour glucose	7	2.42	2.18	±0.82	1.29	0.17	0.132
2-hour fructose	7	2.25	2.63	±0.99			
3-hour fructose	14	13.82	4.95	±1.32	1.64	8.75	5.335
3-hour glucose	14	5.07	3.65	±0.98			
Index of glycogen formation in muscle							
1-hour glucose	11	0.327	0.379	±0.114	0.211	0.216	1.024
1-hour fructose	13	0.111	0.637	±0.177			
2-hour glucose	16	0.661	0.394	±0.099	0.129	0.202	1.566
2-hour fructose	17	0.459	0.340	±0.082			
3-hour glucose	15	0.687	0.307	±0.079	0.101	0.043	0.426
3-hour fructose	14	0.644	0.237	±0.063			
Per cent of absorbed sugar deposited as muscle glycogen							
1-hour glucose	11	16.36	18.96	±5.72	10.35	10.78	1.042
1-hour fructose	13	5.58	31.87	±8.84			
2-hour glucose	16	33.06	19.66	±4.92	6.41	10.11	1.577
2-hour fructose	17	22.95	16.98	±4.12			
3-hour glucose	15	36.12	12.95	±3.34	4.60	3.92	0.852
3-hour fructose	14	32.20	11.85	±3.17			

in order to accomplish the disposal of the products of muscle metabolism. Moreover, probably some period of time is required for the liver to return to its optimal conditions for glycogen formation.

The greater lag shown by fructose may indicate that certain time-consuming rearrangements of the molecule must occur before it can be polymerized to the polysaccharide. Other factors may be involved, such as the lower renal threshold for fructose, a more rapid conversion of fructose to fat, and a greater ease of oxidation of fructose. The conclusion, arrived at in earlier investigations, that fructose is superior to glucose as a glycogenic agent, has generally been based on analyses of the liver made several hours after administration of the sugars, without regard for the immediate effects or the concurrent changes in the muscle.

Why fructose should produce a higher maximum of liver glycogen than glucose, of which there is more available, can hardly be decided on the basis of our present knowledge. It might be postulated that the polymerization occurs first in the liver, and that the glycogen deposited from fructose is less readily hydrolyzed than that from glucose, for transfer to other parts of the body. However, there is at present no evidence of chemical or biological differences in the glycogens formed from the two sources.

SUMMARY AND CONCLUSIONS

Rats, fasted and fatigued by swimming were fed glucose or fructose solutions by stomach tube. After 1-, 2- and 3-hour absorption periods the animals were anaesthetized with amytal, liver and muscle samples obtained, and the unabsorbed residues in the gastrointestinal tract were collected. The amount of sugar absorbed, and the increases in liver and muscle glycogen above the pre-formed control level were determined. Fructose was found to be absorbed at a somewhat slower rate than glucose by the fatigued rat, but both sugars showed a falling off in absorption rate as the time was prolonged. To allow for the inequalities in rate of absorption,

the new glycogen formation in liver and muscle from glucose and fructose was compared on the basis of the "index of glycogen formation," which takes into account both absorption rates and glycogenesis rates, as well as on the basis of percentage of the total amount of sugar absorbed that is deposited in the total liver and muscle glycogen stores. Glucose was found to be superior to fructose in rebuilding the liver glycogen stores during the early stages, i.e., the first hour, of recovery after exhausting fatigue, the difference being statistically significant. Fructose approaches glucose in activity in the liver in the second hour, and in the third hour is much more effective than glucose. In muscle the average results for new glycogen formation indicate a greater effectiveness for glucose, especially in the first 2 hours, but the difference between the two sugars is not statistically significant at any time.

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STUDIES ON THE VITAMIN C METABOLISM OF FOUR PRESCHOOL CHILDREN [1]

MILICENT L. HATHAWAY AND FRIEDA L. MEYER

New York State College of Home Economics, Cornell University, Ithaca

There have been very few reports of quantitative studies on the vitamin C requirements of healthy preschool children. Everson and Daniels ('36) reported a study of three preschool boys, for twelve 5-day periods on levels of 50 to 214 mg. intake. They considered that 6 to 7.5 mg. per kilogram were required to give optimal retentions in these children. Widenbauer ('37) found that the "Zusätzlichen Tagesverbrauches" value for a normal 2½-year-old boy averaged 21 to 22 mg. per day for seven weekly periods. Other studies on the vitamin C requirements of preschool children have been short-time investigations on larger numbers of convalescent children in hospitals rather than detailed studies on a few healthy ones, and differences in the methods employed make comparison difficult. Several of these studies will be discussed later in this report.

Because of wide differences in the requirement reported by the various workers, it seemed important to carry out further studies on the needs of the normal preschool child.

EXPERIMENTAL

Subjects. Four healthy preschool children, two boys and two girls, were selected for this study. Physical examinations given at the beginning and at the end of the experiment showed them all to be normal according to present standards

of measurement.   Data on their sex, age, weight, height, placement on Grandprey's ('33) charts and basal metabolic rate at the end of the experiment are given in table 1. This period was selected to be reported since the basal tests were made at this time.

TABLE 1

Physical status of subjects

SUBJECT	SEX	AGE	WEIGHT	HEIGHT	GRANDPREY'S PLACEMENT FOR			BASAL METABOLIC RATE		
					Wt. for age	Ht. for age	Wt. for ht.	Cal-ories	Deviation from standard for	
									Weight [1]	Height [1]
		mo.	kg.	cm.	%	%	%		%	%
D.T.	M.	64	17.7	106.7	50	45	70	791	—1	—3
J. S.	F.	59	20.6	109.7	over 90	85	over 90	763	—11	—5
. C. M.	F.	43.7	17.8	105.4	over 90	over 90	85	830	+4	+7
D. S.	M.	43.2	16.8	99.1	85	70	90	856	+11	+10

[1] See Talbot's Standards for children ('38).

Caloric intake. During the first weeks of the study an attempt was made to give the two older children, D. T. and J. S., 25% more food than the younger ones.   However, it was difficult to get them to take it, and consequently the amount of food given was cut to the lower level for all four children.   The average daily caloric values for the food actually eaten were calculated from Waller's tables ('35).   The following values represent the daily averages for the last 3 months of the experiment, the period covered in detail by this report: D. T., 1218 Calories; J. S., 1217 Calories; C. M., 1235 Calories, and D. S., 1230 Calories.   On the basis of the basal metabolic tests it is not surprising that the older children did not want more food, since their basal requirement was lower in both cases, and their activity was about the same as that of the other two children.

Diet. The basal diet met the recommended optimal standards in all known nutrients except vitamin C.   The foods used and their vitamin C content are given in table 2.   The food other than milk contained 3 to 6 mg. ascorbic acid per day, and an additional 10 to 14 mg. per day, depending on the sample,

were supplied by the 800 cc. of specially prepared milk used in the study [2]. This milk which was furnished by the Dairy Department of Cornell University, had the oxygen removed by a method developed by Professors Sharp, Hand and Guthrie ('39), a process which preserves the ascorbic acid content and prevents the development of any oxidized flavor. The milk was prepared weekly by Dr. Guthrie and samples were tested by him for ascorbic acid and flavor at the beginning and end of the week to check its keeping quality. Canned fruits, vegetables, salmon and chicken were used, and all foods were bought in quantity whenever practicable. Several analyses were made of each food included in the diet to check the vitamin C content and little variation was found between the analyses. Average values were used in table 2. All menus were repeated weekly. Crystalline ascorbic acid [3] was added to the basal diet at levels from 200 mg. per day down to 15 mg. per day. The ascorbic acid was given with the milk and the additions were regulated so that not more than 50 mg. were added at any one time, e.g., the test dose of 200 mg. was given in 50 mg. portions in the milk at mealtime, and following the afternoon nap.

Urine collections. Each urine specimen was separated into two equal portions. One, which was used for the vitamin C determinations, was placed in a brown bottle and acidified immediately with 10% by volume of 2 N sulfuric acid containing 2% metaphosphoric acid. The other was preserved with thymol for pH determinations and mineral analyses. All samples were placed in the refrigerator as soon as preserved.

Vitamin C determinations. Daily determinations of the vitamin C output were made on 24-hour samples of the acidified urine. The following modifications were made in Bessey's method ('38) with the Evelyn photoelectric colorimeter. To

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[2] The vitamin C values of the milk reported here are 3 mg. per liter lower than those reported by Sharp et al. for the same milk due to the difference between values obtained by titration and by the Evelyn photoelectric colorimeter.

[3] Hoffman LaRoche.

TABLE 2

Basal foods and their vitamin C values

FOOD	AMOUNT	VITAMIN C		DAYS PER WEEK FOODS WERE USED
	gm.[1]	*mg./100 gm.*	*mg./sample*	
Cereals				
Bread	36	...	...	7
Triscuits	16	...	...	7
Ralston	60	...	...	2
Oatmeal	60	...	...	3
Shredded Ralston	20	...	...	2
Rice	32	...	...	6
Macaroni	32	...	...	1
Meats				
Beef	32	0.92	0.29	4
Salmon	32	0.0	0.0	1
Chicken	32	0.71	0.23	1
Veal heart	32	1.58	0.51	1
Vegetables				
Beets	32	3.08	0.99	3
Peas	32	6.25	2.00	3
Carrots	32	0.97	0.31	4
Beans (Wax)	24	3.07	0.74	4
Potato	32	3.16	1.01	7
Fruits				
Prunes	52	0.53	0.28	3
Prune juice	8	1.43	1.03	3
Applesauce	60	1.79	1.07	4
Peaches	52	1.58	0.82	3
Peach juice	8	2.00	0.16	3
Pears	52	1.06	0.55	4
Pear juice	8	1.44	0.12	4
Egg	32	...	...	7
Milk	800 cc.	13–17/liter[2]	10–14	7
Cod liver oil	1 tsp.	...	...	7

Curd free butter[3], sugar, plum and quince jelly were given ad libitum

[1] All weights are for cooked food.

[2] See text.

[3] Prepared for us by Dr. E. S. Guthrie, Professor of Dairy Industry, Cornell University.

samples of urine preserved as indicated above, equal volumes of 2% metaphosphoric acid instead of 6% metaphosphoric acid were added. The H_2SO_4 used to preserve the urine did not interfere with the vitamin C determinations on addition of the 2% metaphosphoric acid. Duplicate solutions were prepared for each sample and triplicate readings made on each. The average of these six readings was used for the "Gs" and "Gsr" values. The "Gb" values were determined for the respective "Gsr" averages at the end of all analyses, using one tube of dye and blank. The value for K_1 as determined for our colorimeter was found to be 0.088 ± 0.002.

For the food analyses, Bessey's method ('38) was followed.

Although Chinn and Farmer ('39) showed that the feces of adults contain about 5 mg. of ascorbic acid daily, they found that in the normal individual large variations in the dietary intake affected the fecal excretion only slightly. Consequently no attempt was made to measure this excretion.

Plan of the experiment. Through the employment of graduate students trained in child care and the cooperation of the Family Life Department of the College, every effort was made to give the children happy normal experiences throughout their stay in the laboratory.

A preliminary period of about 10 days was used in which the children became adjusted to their new surroundings and new companions. During this period they learned to eat the foods included in the diet, to clean their plates, etc.; and they were given 50 mg. per day of crystalline ascorbic acid in addition to that in the food to insure tissue saturation.[4] The early periods on the 66 mg. level noted in table 3 were omitted in table 4 since they were of only 1-week duration and showed results almost identical with those obtained on the 2-week periods.

[4] The term "saturation" as used throughout this report refers to the condition in which the tissues have taken up from the blood stream all the vitamin C which they are capable of storing, so that on a constant high intake of the vitamin a relatively large and constant amount is eliminated in the urine.

Throughout the study each period was concluded by a test dose of 200 mg. to check tissue saturation. The periods were of either 1 or 2 weeks duration as indicated in table 4. In computing averages the first 2 days following a test dose were omitted to eliminate the excessive excretion resulting from the larger intake.

These results represent a total of thirty-two of the periods studied, eight for each child.

TABLE 3

Twenty-four hour excretion of ascorbic acid in response to 200 mg. test dose following periods on various levels of ascorbic acid intake

DAILY INTAKE OF ASCORBIC ACID	D.T.	J.S.	C.M.	D.S.	AVERAGE
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
115	162	141	150	123	144
92	133	142	138	144	139
66	130,129,118	119,114,133	127,141,146	132,112,132	128
50	130	114	140	129	128
42	112+	138	132	143	131
31	107,109	73,112	113,119	89,119	105
17	83	72	....	50	68

RESULTS

The urinary responses to 200 mg. test doses following 1 or 2-week periods on various levels of ascorbic acid intake are shown in table 3. If an excretion of 50% of the intake is considered as the criterion of saturation, it is evident that on an intake of 115 mg. down to 42 mg. per day the tissues were saturated in all the children, but that on the 31 mg. level it was only following the second period at this level that D. S. and J. S. were saturated. At the 17 mg. level the tissues were not saturated in any of the children. It might be said, then, on the basis of their responses to test doses, that a daily intake of 31 mg. appears to be the marginal level for saturation in these children.

Table 4 summarizes the results for the average daily urinary responses of the four children on the various levels of ascorbic acid intake. During any week represented by these data,

except the week on the 115 mg. level, the variation in ascorbic acid excretion for any of the children averaged 6 mg. with a standard deviation of $\pm$ 3 mg. Variations at the highest level (115 mg.) were from 13 to 37 mg.

TABLE 4

Average daily excretion and "utilization" of ascorbic acid on various levels of intake

PERIOD	1	2	3		4		5		6		7		8
INTAKE IN MG.	115	92	66	66	50	50	41	43	32	31	31	32	17
Urinary excretion in mg.													
D. T.	77	59	38	32	26	20	17	19	9	11	14	11	6
J. S.	82	46	37	33	25	24	15	16	9	7	8	10	5
C. M.	90	61	39	39	29	27	18	20	11	7	10	10	6
D. S.	74	66	35	33	26	25	18	16	10	8	9	10	5
av.	81	58	37	34	26	24	17	18	10	8	10	10	5
"Utilization" in mg.													
D. T.	39	33	28	34	25	30	24	24	23	20	17	21	11
J. S.	34	46	29	33	26	26	26	27	23	24	23	22	12
C. M.	26	31	27	27	22	23	23	23	21	24	21	22	11
D. S.	42	26	31	33	25	25	24	27	22	23	22	22	12
av.	35	34	29	32	24	26	24	25	22	23	21	22	11
"Utilization" in %													
D. T.	34	36	42	51	49	60	58	56	72	64	55	66	65
J. S.	29	50	44	50	51	52	63	63	72	77	74	69	71
C. M.	22	34	41	41	43	46	56	53	66	77	68	69	65
D. S.	36	28	47	50	49	50	56	63	69	74	71	69	71
av.	30	37	43	46	46	52	58	59	70	73	67	68	68

Table 4 also includes the values for the "utilization" of the ascorbic acid. This term "utilization" is used arbitrarily to refer to the difference between intake and excretion. The mean value for the "utilization" level for the four children on intakes of 31 to 50 mg. is 23 mg. with a standard deviation of $\pm$ 2 mg. Thus the "utilization" on the 31 mg. level is about 74% of the intake, but on the 50 mg. level it is only about 47% of the intake. In no case was the average efficiency during any given week greater than

77%. On the basal diet alone the "utilization" did not exceed this figure although the tissues were not saturated. This may be considered another evidence that a daily intake of about 30 mg. is the marginal level required for saturation, since at this level the children were able to maintain tissue saturation with the maximum efficiency in the utilization of the ingested ascorbic acid.

DISCUSSION

Workers who have studied the vitamin C metabolism of children differ markedly in their criteria for tissue saturation. Two groups, Keith and Hickmans ('38) and Parsons ('38), used 500 mg. as the test dose, and considered the excretion of 50 mg. in the next 24 hours as the criterion of saturation. This seems a very low percentage in the light of data reported here for much lower intakes of the vitamin. Both groups determined the vitamin C by titration with 2–6 dichlorophenolindophenol.

Widenbauer ('37) considered that on an intake of 200–500 mg. determined by body weight and expected deficiency, saturation was reached when at least 50% of the half-day dose was excreted in 12 hours. He also used the titration technic, but his test dose and percentage values correspond more closely to those in this report.

Probably the most thorough study on the effect of the differences in the size of the test dose and the criteria of saturation is found in Baumann and Rappolt's report ('37). They showed that using 25 mg. per kilogram (or about 500 mg. for children of preschool age) no vitamin C deficit existed if 29–59% of the intake was excreted within 24 hours. In one subject on a 200 mg. intake 43% excretion in 24 hours was considered indicative of saturation whereas on a 100 mg. test dose the same subject excreted 90% in the same length of time. They found much greater variation on the higher test doses. It should be mentioned that the vitamin C content of the diet was not considered in their figures, and that the titration method was used.

No quantitative studies on the requirements for tissue saturation in children have been found in which the photoelectric colorimeter was used for the determination of vitamin C. Therefore it was necessary to set up our own criterion for saturation. Belser, Hauck and Storvick ('39), who studied saturation in adult subjects using the titration technic, allowed each individual to set his own standard for comparison. However, it will be noted that at least 50% of the test dose of 400 mg. was excreted when their subjects were saturated. By referring to table 3 it can be seen that if the individual response to the test dose following a period on 66 mg. is taken as the standard for comparison in these four children, all but D. T. equal or pass this standard down to the 31 mg. level. The value 112 for D. T. may be lower than it should be, due to a slight loss in his morning specimen in the laboratory before analysis. On this basis, one test on D. S. and J. S. would still show saturation on the 31 mg. level, although D. T. and C. M. would have values slightly below saturation at this level. Further tests will be made to establish if 50% excretion is a fair test for saturation, but since the above comparison with the method of Belser, Hauck and Storvick ('39) shows similar results, the conclusion that a daily intake of about 30 mg. is the marginal level for tissue saturation seems valid on either basis.

The term "utilization" is used arbitrarily to cover that portion of the ascorbic acid ingested which is not excreted in the urine. It is conceded that a part of this is probably excreted in the feces (Chinn and Farmer, '39), part may be decomposed in the gastrointestinal tract or within the body after it is absorbed, and part may replace some of the vitamin stored in the body, but that amount seems necessary for bodily function.

In this experiment little change was found in the amount of ascorbic acid "utilized" on intakes ranging from 30 to 50 mg. The question arises whether the increased "utilization" noted on higher levels is real or apparent, i.e., there might be a greater destruction of the vitamin at these higher levels. Better tests for measuring function and partition of

vitamin C within the body will be necessary before this question can be answered.

Comparison of the figures in the present study with those of Everson and Daniels ('36) at the 50 mg. level shows interesting differences. The present "utilization" figures were 22–30 mg., those for Everson and Daniels 36–40 mg. These differences may easily be explained on the basis of previous saturation of the tissues in this experiment. The importance of this factor had not been recognized when the earlier work was done. It was only when the intake levels reached 200+ mg. that the excretion values were in the range found in the present study. This might suggest that in the earlier study it was only when this level of intake was reached that the tissues were saturated. Since the tests on the 200+ mg. levels were the last ones made there was no check on excretion values following saturation.

It is of interest to note also that the greatest average efficiency of retention for any 5-day period in Everson and Daniels' tests was 77% for G. E. on a 50 mg. level on 12/15/36, the same value found by us with J. S. and C. M. on the 31 mg. level. Further observations on more children are needed before conclusions can be drawn but it can be stated that in these studies on seven preschool children over a 5–6 month period the efficiency of "utilization" or retention of ascorbic acid was not greater than 80% of the vitamin ingested regardless of the intake level. In four of the children the tissues were saturated except on the lowest level fed, but in the other three they were probably somewhat below saturation.

In contrast to this work with children the studies of Kellie and Zilva ('39) and Ralli, Friedman and Sherry ('39) with adults may be cited. Kellie and Zilva found that if the tissues had been saturated previously the retention on a 50 mg. daily intake was 70%, but if a state of "unsaturation" existed as much as 90% was retained even after 17 days at this level. Ralli et al. found retention values averaging 87 to 94% over a period of 140 days for one subject, and of 86 to 92% over a period of 127 days for a second one. This might indicate that in adults the ability to conserve (or destroy) ascorbic acid is greater than that found in children.

Although Widenbauer ('37) does not give the details of his experiment, our figures for vitamin C requirement are in essential agreement with his. He concluded that 22 mg. ascorbic acid were needed daily by a normal 2–3-year-old boy in addition to the experimental diet, low in vegetables and free from fruits. Our figure of 31 mg. per day would allow 9 mg. in his diet which is a plausible figure.

The requirement for vitamin C in these four children does not seem to be related to sex, as D. T. and D. S. were boys, J. S. and C. M. girls; nor is it affected by age at least within the range of 15 to 20 months covered by our subjects. It does not seem to be related to body weight, as their weights varied 3.5 to 4 kg. throughout the experiment. C. M.' gained weight much more rapidly than the other children, increasing 2.5 kg. as compared with 0.5 to 1 kg. each for the other three children, but she showed no higher retention of vitamin C than the rest at any period in the study. Ascorbic acid requirements showed no correlation with the basal metabolic rates. The two children showing unsaturation after the first period on 31 mg. differed in age by 15 months, in weight by 4 kg. and in B.M.R. from $+11\%$ for the younger boy to -11% for his older sister. Ralli et al. ('39) also concluded that weight did not affect the vitamin C requirement as A. T. gained 20 pounds during the 200 days on the experiment with no effect on the vitamin C needs.

SUMMARY AND CONCLUSIONS

Four preschool children were studied on various levels of ascorbic acid intake in an otherwise constant diet. After it was established that their tissues were saturated with vitamin C by checking their responses to test doses of 200 mg. of pure ascorbic acid, the level of intake was varied as follows: 115, 92, and 17 mg. were used for 1-week periods, and 66, 50, 43, and 31 mg. for 2-week periods. The 31 mg. level was repeated. Each period on a new level was followed by a test dose to recheck tissue saturation. The results of the study may be summarized as indicated below.

1. A daily intake of 31 mg. of ascorbic acid was the marginal level to insure tissue saturation in these four children.

2. The "utilization" of ascorbic acid was relatively constant at levels of intake from 31 to 50 mg. and the average value for these four children for four 2-week periods was 23 ± 2 mg.

3. The per cent retention or "utilization" found in these children was lower than those reported in the literature for adults.

4. The requirement was not related to sex, age or body weight, at least within the limits found in these children.

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THE BIOLOGIC RESPONSE OF CHICKENS TO CERTAIN ORGANIC ACIDS AND SALTS WITH PARTICULAR REFERENCE TO THEIR EFFECT ON OSSIFICATION

JOHN T. CORRELL

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

Interesting developments are now appearing in the literature as a result of continued studies on the relation of acid and alkaline-ash foods to the etiology of rickets and the metabolism of vitamin D.

The earlier work on this problem has been presented and summarized by Shohl et al. ('28, '31, '32). Among other things it has been reported that the cation-anion balance of the blood is normal in clinical rickets, infantile tetany and, except in special cases, in experimental rickets. Later work indicated that certain cationogens and anionogens have, however, a marked effect on such pathologic states. Hamilton and Schwartz ('33) reported that rickets was completely prevented in rats on a rickets-producing diet by the addition of the cationogen, acid sodium tartrate. In rats on a non-rachitogenic diet rickets was produced by a mixture of the anionogens, ammonium chloride and ammonium carbonate. Hamilton and Dewar ('37) extended this investigation and demonstrated that sodium citrate, sodium bitartrate, citric acid, and tartaric acid all prevented the development of rickets in rats maintained on a rachitogenic diet.

The principal conclusion from the above mentioned researches, that some organic acids and the salts of these acids when fed to rats on a rachitogenic ration will permit normal ossification even in the absence of any form of vitamin D,

has been verified by Hathaway and Meyer ('39), Day ('40) and Shohl ('37). This latter author fed eight basal diets, each one having a different calcium to phosphorus ratio, and found that ammonium chloride-ammonium carbonate mixtures produced rickets with rations which previously were non-rachitogenic or intensified the rickets produced by the rachitogenic feeds. Citric acid-sodium citrate mixtures added to rachitogenic diets protected against rickets. Several organic acids were tested but only the citrates and tartrates were found to be effective and these were more beneficial when used as a mixture of the acid and its sodium salt.

Most of these investigations were carried out using the white rat as an experimental animal. Endeavors to apply this information to human subjects have been necessarily limited. Publications by Hanke ('33) and Chaney and Blunt ('25) have shown that in the human being there is a definite retention of calcium and phosphorus after the inclusion of large amounts of orange juice in the diet. Lanford ('39) suggested that the beneficial effects of orange juice, which she too noted, might be due to the citric acid and citrates in such a supplement. Shohl and Butler ('39) tested the value of organic acid therapy on two cases of human rickets. They concluded that mixtures of citric acid and sodium citrate induced healing in infantile rickets without vitamin D supplement.

A search of the literature has failed to reveal the use of any other laboratory subject besides the rat and dog in studying this interesting problem. Within the last few years the chick has become a popular biologic tool to demonstrate differences between the various forms of vitamin D. Thus, Massengale and Nussmeier ('30) and others have demonstrated that chick bone ash is more sensitive to vitamin D in cod liver oil than to that in irradiated ergosterol when used in equal units as standardized on the rat. Likewise Correll and Wise ('38 a, '38 b) found the growth of the chick and the phosphatase concentration in the serum to be

affected by the type of antirachitic agent incorporated into the ration.

It seemed of interest to try and find out if these organic acid-salt mixtures when fed to the chick would produce normal bone ossification even in the absence of vitamin D, or if ammonium chloride-ammonium carbonate would produce rickets with a non-rachitogenic diet as these supplements had been shown to do in the rat. If such results were obtained, what would be the effect on the phosphatase concentration which, supposedly, is directly affected by vitamin D intake? The chick lends itself admirably to further investigation of this problem for, as Massengale and Bills ('36) have pointed out, the production or non-production of rickets in the chick is not dependent upon a favorable mineral ratio. Variations in the calcium-phosphorus content of the diet do not result in an appreciable difference in the bone ash values obtained under a given set of conditions.

It is the purpose of this paper to present the results of our research on these questions.

EXPERIMENTAL

Single comb white Leghorn chicks were started on assay when 1 day old (weight 30 to 35 gm.) and the experiments terminated on the twenty-first day. The birds were divided into groups of fifteen or twenty without regard to sex, each group being kept in a wire-bottomed, electrically-heated battery brooder with constant access to distilled water and the ration.

The basal diet employed was that of Hart, Kline and Keenan ('31), modified to contain fifty-seven parts of maize and two parts of dried yeast. Numerous analyses of this ration in our laboratory have shown it to contain about 1% calcium and 0.7% phosphorus; $\frac{Ca}{P}$ ratio of $\frac{1.4}{1}$. Supplements were made at the expense of the mixed ration as a whole and incorporated so that 100 gm. of feed contained the desired quantity of material to be tested.

Ash determinations and the technique for estimating phosphatase values were carried out according to the methods referred to in previous work (Correll and Wise, '38 a, '38 b).

Every experimental series included negative and positive control groups. This allows for comparison of the results yielded by any group in a series with data from chicks which were rachitic and those that were normal under the conditions of each experiment. It has been noted before (Correll and Wise, '38 a) that bone ash values vary from time to time even under the most uniform conditions. Such fluctuations are of no importance in the interpretation of these tables because positive and negative controls were utilized as indicated.

Since an assay group represents either fifteen or twenty birds, results are recorded as an average of the whole for conservation of space.

<center>RESULTS</center>

The first experiment was designed simply to observe the effects on the bone ash of chicks of certain citrate mixtures when incorporated into a diet known to be rachitogenic for the birds. The salt and acid supplements were identical with those which other investigators had shown to be antirachitic for rats. The quantities fed were planned to approximate the amounts found effective in rats of similar starting weights (30 to 35 gm.).

The results obtained (table 1, experiment I) were unexpected. The only group (citric acid-potassium citrate, group 3) in which a large percentage of the birds lived through the assay period showed poorer growth than the positive controls and complete failure of the supplement to enhance ossification as judged by the bone ash value obtained. The other acid-salt additions, which had been effective for rats, killed the chicks. The citric acid-sodium citrate appeared to be especially toxic (group 5).

This led to an investigation of a series of cationogen and anionogen mixtures. All of these have been reported in the

TABLE 1

The effect of adding cationogens and anionogens to rachitogenic and non-rachitogenic diets for chicks

GROUP NUMBER	SUPPLEMENT PER 100 GM. OF DIET	BIOLOGIC RESPONSE				
		Final weight	Bone ash	Phospha-tase	Survival data [1]	"Salt" effects
Experiment I						
		gm.	*%*	*Units* [2]		
1	Negative controls	89	36.9	*	18	*
2	Positive controls 27 I.U. of D from C.L.O.	107	45.0	*	18	*
3	0.02 mol. citric acid 0.02 mol. potassium citrate	72	36.3	*	18	*
4	0.04 mol. citric acid 0.04 mol. potassium citrate	62	34.0	*	5 Others died 2nd, 3rd wk.	*
5	0.02 mol. citric acid 0.02 mol. sodium citrate	*	*	*	All died 1st wk.	*
Experiment II					.	
6	Negative controls	90	36.6	165	17	None
7	Positive controls 27 I.U. of D from C.L.O.	136	47.7	52	16	None
8	0.01 mol citric acid 0.025 mol. sodium citrate	*	*	*	All died 1st wk.	+++
9	0.05 mol. tartaric acid 0.03 mol. sodium tartrate	*	*	*	All died 2nd, 3rd wk.	None
10	0.05 mol. maleic acid 0.03 mol. sodium maleate	*	*	*	All died 1st wk.	None
11	0.1 mol. lactic acid 0.06 mol. sodium lactate	*	*	*	All died 1st wk.	+
12	0.1 mol. acetic acid 0.06 mol. sodium acetate	*	*	*	All died 1st wk.	+++
13	0.02 mol. citric acid 0.02 mol. potassium citrate	56	37.0	114	10	None
14	0.02 mol. citric acid 0.02 mol. potassium citrate 27 I.U. of D from C.L.O.	67	41.6	94	11	None
15	0.02 mol. citric acid 0.02 mol. potassium citrate 100 I.U. of D from C.L.O.	98	48.1	35	17	None
16	0.03 mol. ammonium carbonate 0.06 mol. ammonium chloride 27 I.U. of D from C.L.O.	81	45	55	15	None

[1] Twenty chicks started in each group. This column records how many survived, or how rapidly they died.

[2] Bodansky units per 100 cc. of serum.

* Not investigated for this group.

+ Slight "salt" deposits observed in some birds on autopsy.

+++ Heavy "salt" deposits observed in practically all birds on autopsy.

literature as having been fed to rats. Some were effective antirachitic agents, others not. None was reported as toxic to the rat.

The data are tabulated in table 1, experiment II. Citric acid-sodium citrate (group 8) even at a much lower concentration than in the first experiment was extremely toxic to the chick.

Autopsies were performed on these birds and a gross examination made of the internal organs. Heavy, white "salt" deposits were observed in localized areas throughout the body. The femur-tibio-tarsus joint, or knee, was often swollen by a congestion of the precipitate at this juncture. Such findings accounted for the stiff-legged gait noticed in many chicks before death. The pericardium in some cases was so coated as to make the heart appear as a white stone. Irregular patches of white "salt" were seen scattered through the subcutaneous fascia. The kidneys were always affected, appearing as though full of solid material; when cut they were found to contain a milky, gritty, viscous fluid. The ureters were occluded and hard; from them elongated, stone-like pieces were extruded.

The nature of this precipitate has not been established. Stones obtained from the ureters undoubtedly were highly contaminated. Positive qualitative tests were observed for calcium, phosphorus, sodium, potassium, and citrate radical. The material appeared to be practically insoluble in hot or cold water, alcohol, and concentrated HCl, and somewhat soluble in 25% NaOH. One sample yielded 22.8% ash.

The supplements of tartaric, maleic, lactic and acetic acid-salt mixtures (groups 9 to 12 inclusive) also killed the birds, although only with the acetic acid-sodium acetate mixture were appreciable deposits of "salt" noticed.

Again citric acid-potassium citrate (group 13) allowed fair survival and no "salt" was seen in the birds that died. However, this mixture was in no way antirachitic for the chick as demonstrated by the poor growth, low ash, and comparatively high phosphatase results obtained. Indeed such a sup-

plement appeared to interfere with ossification even when cod liver oil was included in the diet. An established adequate amount of vitamin D, under these conditions (group 14), improved the biologic response of the group but still yielded values far below those shown by the positive controls. A large dose of vitamin D (group 15), four to five times the minimum required by chicks, gave only fair growth but the bone ash and phosphatase values were normal.

The anionogens (group 16) added to the basal diet along with vitamin D, failed to produce rickets as measured by the bone ash and phosphatase values. This supplement did not permit good growth; however, it was not rickets-producing for the chick on a non-rachitogenic diet, as a similar supplement has been shown to be for rats on diets which, because of their favorable mineral ratio, were non-rachitogenic for them.

Potassium citrate mixtures were much less toxic than sodium citrate at equivalent levels (compare group 3 with group 5), and even when fed at a greater concentration (group 4) the birds lived longer with no manifestation of "salt" on autopsy. There was also indication that vitamin D "protected" the chicks somewhat from the toxic action which potassium citrate did exert (see group 13, 14 and 15). Such observations gave rise to the question of what influence potassium citrate and vitamin D would have individually and together if included in a ration with the more toxic sodium citrate.

By reference to table 2, experiment III, it will be noted that vitamin D (groups 20 to 23, inclusive) even in massive doses, failed to protect the chick against the toxicity of sodium citrate. Potassium citrate added with the sodium salt in an equal molar quantity (group 24) allowed for normal survival, but this mixture was not antirachitic for the chick as demonstrated by the low bone ash value. Sodium citrate in excess of potassium citrate (group 25) still killed the birds but the "protective" action of the latter salt is noted by the increase in the time of survival and failure to observe

TABLE 2

The effect of feeding vitamin D or potassium citrate with sodium citrate, and of feeding the component parts of sodium and potassium citrates to the chick

GROUP NUMBER	SUPPLEMENT PER 100 GM. OF DIET	BIOLOGIC RESPONSE			
		Final weight	Bone ash	Survival data [1]	"Salt" effects
Experiment III					
		gm.	*%*		
18	Negative controls	130	37.6	14	None
19	Positive controls 27 I.U. of D from C.L.O.	181	47.7	14	None
20	0.02 mol. sodium citrate	*	*	All died 1st, 2nd wk.	+++
21	0.02 mol. sodium citrate 27 I.U. of D from C.L.O.	*	*	All died 1st wk.	+++
22	0.02 mol. sodium citrate 100 I.U. of D from C.L.O.	*	*	All died 1st, 2nd wk.	+++
23	0.02 mol. sodium citrate 500 I.U. of D from C.L.O.	*	*	All died 1st wk.	+++
24	0.02 mol. sodium citrate 0.02 mol. potassium citrate	101	38.0	13	None
25	0.05 mol. sodium citrate 0.02 mol. potassium citrate	*	*	All died 2nd, 3rd wk.	+
26	0.05 mol. sodium citrate 0.02 mol. potassium citrate 27 I.U. of D from C.L.O.	50	40.0	5 Others died 2nd, 3rd wk.	+
Experiment IV					
27	Negative controls	152	38.2	13	None
28	Positive controls 27 I.U. of D from C.L.O.	155	46.4	15	None
29	0.06 mol. sodium chloride	139	38.7	13	None
30	0.12 mol. sodium chloride	122	37.0	8	None[2]
31	0.06 mol. potassium chloride	112	39.0	15	None
32	0.06 mol. sodium chloride 0.06 mol. potassium chloride	125	40.7	14	None[2]
33	0.04 mol. citric acid	77	33.9	14	None
34	0.02 mol. citric acid 0.06 mol. sodium chloride	120	34.5	15	None
35	0.04 mol. citric acid 0.12 mol. sodium chloride	94	34.2	15	None

[1] Fifteen chicks started in each group. This column records how many survived, or how rapidly they died.

[2] Some edema apparent in these birds.

* Not investigated for this group.

+ Slight "salt" deposits observed in some birds on autopsy.

+++ Heavy "salt" deposits observed in practically all birds on autopsy.

any significant "salt" deposits in the dead birds. Potassium citrate and vitamin D together (group 26) were yet more "protective" against sodium citrate; if the vitamin D had been included at a higher level the chicks might all have lived.

Experiment IV in table 2 is self-explanatory. Neither sodium nor potassium as chloride, in the lower concentrations, were particularly toxic to the chick; when they were, no "salt" deposits were seen. None of these salt supplements is antirachitic; the slight increase in bone ash noted in group 32 is probably not significant. Citric acid alone (group 33) did not kill the birds, although neither did it allow for good growth. In group 34 sodium chloride and citric acid were added in amounts calculated to produce sodium and the citrate radical equal to that yielded by 0.02 mol. of sodium citrate. Quantities equivalent to 0.04 mol. of sodium citrate were fed to another group (group 35). Under these conditions no toxicity was observed comparable to that demonstrated when 0.02 mol. of sodium citrate was included in the diet. All the chicks lived, but the salts were not antirachitic as shown by the bone ash percentages.

DISCUSSION

It would appear from these results that probably no simple organic acid or its sodium and potassium salt, when added to a rachitogenic diet, will protect the chick from rickets as it does the rat. From one experiment it also seems that ammonium chloride-ammonium carbonate mixture in a ration non-rachitogenic for chicks will not produce rickets in the birds as it has been shown to do with rats on basal diets that were non-rachitogenic because of a favorable mineral ratio.

Most of the cationogens fed to the chicks were toxic, particularly sodium citrate and acetate which caused the precipitation of a "salt" in the animal's body. Potassium citrate seems to "protect" the chick from this toxic manifestation of sodium citrate. Likewise vitamin D allowed the chick greater tolerance for the somewhat toxic potassium citrate. No explanation is offered for these observed facts.

The author is indebted to Mr. Anthony Bucci for his care of the animals and for assistance in the autopsies.

SUMMARY

1. No organic acid, its sodium or potassium salt, or a mixture of the acid and its salt when added to a diet that is rachitogenic for chicks was found to protect the birds from rickets as similar mixtures are known to do for rats.

2. Most of the cationogens studied were toxic to the chick. Sodium citrate and acetate caused the precipitation of an insoluble "salt" in the animal body.

3. When citric acid and sodium chloride were fed in amounts equivalent to a lethal dose of sodium citrate no such toxic manifestations were noted.

4. Potassium citrate is less toxic than sodium citrate. When the two were fed together in an equal molar ratio the chick was "protected" from the greater toxicity of the sodium citrate.

5. Ammonium carbonate-ammonium chloride mixture when added to a non-rachitogenic ration for chicks did not produce rickets, as measured by bone ash and serum phosphatase values, in contrast to results that have been reported for rats on diets which were non-rachitogenic because of a favorable mineral ratio.

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A STUDY OF VITAMIN C NUTRITION IN A GROUP OF SCHOOL CHILDREN

PART II. DIETARY EVALUATION [1]

ELIZABETH MURPHY

Maine Agricultural Experiment Station, Orono

ONE FIGURE

A study of the vitamin C status of a group of eighty-six elementary school children in a Maine village was made during the late fall of 1938 and early spring of 1939, seasons when vitamin C containing foods are less abundant than during the summer months. Most of the children were of French-Canadian extraction, and the dietary habits, food preferences, and cooking methods are characteristic of the native stock of this region only.

The following criteria were used to judge vitamin C nutrition: (1) fall and spring plasma ascorbic acid values as determined by a modification of the Ingall's technique ('37); (2) the results of urinary tolerance tests on forty-nine children, twenty-three of whom were from the group of eighty-six children; (3) oral conditions determined by examination in fall and spring; (4) the effect of vitamin C therapy on gum inflammation among forty-one children, twenty-six of whom were from the group of eighty-six children; (5) analyses of dietary records kept for 1 week by seventy-six children in the autumn and sixty-three children in the spring of whom

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sixty and forty-six respectively were from the group of eighty-six children; [2] the record of only one child in a family was used giving one record for approximately every five of the 325 families represented at the school.

The children in the study were unselected except for age and sex. The group included thirty-nine boys and forty-seven girls, ranging in age from 7 to 16 years.

The laboratory tests involving the determination of plasma ascorbic acid and urinary tolerance tests were conducted by Dr. Marian M. Crane of the Children's Bureau, Department of Labor, Washington, D. C. The oral examinations were made by Dr. Phillip W. Woods of the Dental Division, Bureau of Health, Augusta, Maine. It is pertinent to the present report to summarize their results briefly as follows:

RESULTS OF THE CLINICAL AND LABORATORY STUDIES [3]

1. Approximately 45% of the children in the autumn and nearly 63% in the spring manifested plasma ascorbic acid values of less than 0.40 mg. per 100 ml. Only two children of the group manifested values of 0.80 mg. per 100 ml. or above at both autumn and spring examinations.

2. The urinary tolerance tests demonstrated that the twenty-eight children whose plasma ascorbic acid values were less than 0.40 mg. per cent all excreted less than 10% of a 400 mg. dose of ascorbic acid within 6 hours after ingestion. In contrast, the four children with plasma values 0.80 mg. per cent or above excreted more than 10%, in three cases 30% or above.

3. Oral examinations showed gum inflammation in slightly more than one-third of the children in the autumn and about one-half of the children in the spring. Of the twenty-four

[2] A number of additional records obtained in the spring could not be included because the families had recently received grapefruit from the Federal Surplus Commodities Corporation. The records were, therefore, not considered to represent the child's usual diet.

[3] Crane, Marian M., and Phillip W. Woods. 1941. A study of vitamin C nutrition in a group of school children. Part I. Clinical and laboratory studies. New England J. Med., vol. 224, p. 503.

children with low plasma ascorbic acid values (below 0.40 mg. per cent) both in autumn and spring, only three were free from gum inflammation at both examinations. Of the seventeen children with consistently higher plasma values (0.40 mg. per cent or above), ten were entirely free from inflammation.

4. Of the forty-one children included in the therapy test, six showed extensive, nineteen moderate, and sixteen slight gum inflammation. The children were instructed to take orally 200 mg. of crystalline ascorbic acid daily, and were re-examined from 19 to 21 days after therapy was started. About two-thirds (twenty-eight) of the forty-one children showed improvement in oral condition, thirteen being free from inflammation. About one-third (thirteen children) showed no change in oral condition.

DIETARY EVALUATION IN TERMS OF VITAMIN C

Food records

Concurrent with the observations reported above on plasma ascorbic acid values and oral conditions of the school children, a study of dietary habits and food values was carried out.

The frequencies with which vitamin C containing foods (except potato which is discussed in a separate section) were reported is shown graphically in figure 1.

Of the foods commonly eaten in the United States, citrus fruit is the best source of vitamin C. In the autumn only seventeen of the seventy-six children ate any citrus fruit during the week for which the record was kept; in the spring half of the children had some citrus fruit but only fourteen of the sixty-three had it oftener than once or twice in the week.

Other foods reported which are good sources of vitamin C [4] are tomatoes, cabbages, and turnips (rutabaga). These foods,

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[4] Greens, which are usually considered a good source of vitamin C, have not been included as such here. Chemical analyses of greens, after preparation for consumption according to the local methods, showed their vitamin C content to be relatively low.

as well as citrus fruits, were lacking from the diets of fifteen of seventy-six children in the autumn and ten of sixty-three children in the spring, and they were eaten only once or twice a week by twenty-nine children in the autumn and fifteen in the spring. At neither season did more than one child in seven have one good source of vitamin C each day, the minimal recommendation for a satisfactory diet (Children's Bureau Folder, '39).

FREQUENCY WITH WHICH VITAMIN C CONTAINING FOODS WERE CONSUMED
(76 RECORDS IN AUTUMN, 63 RECORDS IN SPRING)
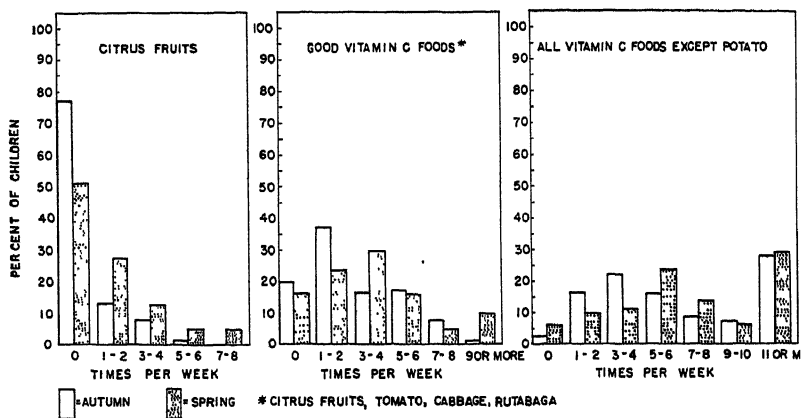
Figure 1

The other foods supplying vitamin C that were included in the diets studied are only fair sources of this factor. There were at least half of the children (forty-three of seventy-six in the autumn, thirty-two of sixty-three in the spring) who did not eat any vitamin C containing food other than potato as often as once a day and about one-sixth who did not eat such food more than twice during the week.

Figure 1 shows that many of the diets were markedly inadequate in respect to vitamin C containing foods. Only 14% in the spring and 9% in the fall included one food which is a good source of vitamin C daily, while 40% in the spring and 58% in the fall reported such a food only twice a week or even less.

Food analyses

While conducting this vitamin C survey in the State of Maine, it was found necessary to perform a certain number of analyses for vitamin C in order to evaluate the diets of these subjects. In the first place, many of the foods reported were substances whose vitamin C contents had never been determined and which seemed unique to the area. Secondly, cooking methods in that particular district differed from usual techniques and the data for cooked foods found in the literature were not applicable to the foods consumed in the district. Thirdly, investigations at the biological laboratory of the Maine Agricultural Experiment Station had shown that locally-grown produce varied considerably as to vitamin C values from that produced elsewhere (Murphy, '39).

METHOD

To investigate destruction of vitamin C by home cooking, a frequency distribution was made of the cooking technique data obtained on visits to each home. These data included length of cooking time, amount of water used, type of cooking utensil, and method of preparation of vegetables before cooking.

On the basis of this information, several of the homes where typical methods were used were visited. Procedures were observed with a view of duplication in a fully equipped laboratory. Samples of the cooked foods [5] were taken to the temporary field laboratory and analyzed for reduced ascorbic acid using the Bessey and King ('33) titrimetric method.

When duplicate analyses were performed at the permanent laboratory at Orono, it was found that all of the foods under observation contained some of the available vitamin C in the dehydro form. For that reason and for purposes of brevity, most of the data obtained in the field laboratory on reduced ascorbic acid are not included in this paper.

[5] Chemical analysis of the cooking water was omitted, since, in the homes, it was always discarded.

EXPERIMENTAL RESULTS

Potatoes. The frequency with which potatoes were eaten by the individuals under observation and the rarity of other vitamin C containing foods in the diets place this vegetable in the position of a primary source of vitamin C for this particular region. Following is an analysis of the diet records.

NUMBER OF TIMES PER WEEK POTATOES WERE EATEN	PERCENTAGE OF DIET RECORDS LISTING POTATOES WITH SPECIFIED FREQUENCY	
	Autumn (76 records) %	Spring (63 records) %
0– 4	9.2	15.9
5– 9	68.4	71.4
10–13	22.4	12.7

Thus the losses of ascorbic acid sustained during cooking and storage, while small in themselves, constitute an important loss to the consumer, who is relatively dependent upon this supply.

Locally grown Green Mountain potatoes stored under the usual storage conditions of the region and cooked according to the local custom were analyzed for total ascorbic acid using the technique published by Reedman and McHenry ('38). Reductions with H_2S were carried out on all samples, raw and cooked, as this was found to be necessary—the per cent of dehydro ascorbic acid varying from 16% to 68% of the reduced. The data are tabulated in table 1.

Cabbage. Cabbage is an excellent source of vitamin C. It may be grown readily in all parts of Maine and may be stored for winter consumption. Of even more importance, it is a familiar food which should require no educative encouragement for its inclusion in the dietary. It was chosen by only nineteen of ninety-one children in the spring and by eighteen of seventy-six children in the fall. Of these, four of the fall group and five of the spring group consumed it raw.

Varietal differences in Maine grown cabbages were found to be of interest. As nearly as could be determined by inquiry, the Ballhead varieties are the most commonly grown in the region. In analyzing for varietal differences, fourteen varieties

TABLE 1

Ascorbic acid content of fresh foods reported on dietaries. All foods were cooked in covered utensils and in large amounts of water except as otherwise noted

MATERIAL AND SAMPLING TECHNIQUE	METHODS OF PREPARATION	TOTAL ASCORBIC ACID MG. PER GM.		PER CENT RETENTION OF ORIGINAL RAW VALUE
		Raw	Cooked	
Potato. Green Mountains	Raw	0.17		
Fresh, locally grown. Fall	Unpeeled. Boiled 30 min.		0.10	59
Radial sectors from three potatoes	Peeled. Boiled 30 min.		0.10	59
Stored 8 months in local	Raw	0.11		
potato house. Spring	Unpeeled. Boiled 30 min.		0.06	55
	Peeled. Boiled 30 min.		0.05	45
	Baked 400°F. 39 min.		0.07	64
	Fried raw 15 min.		0.08	73
Cabbage. Bugner Ballhead variety. Locally grown	Raw	0.70		
Raw samples from center cross sections of three cabbages				
Cooked samples from quar-	Boiled 30 min.		0.38	54
tered sections of three	Boiled 1 hour		0.31	44
cabbages	Boiled 2 hours		0.23	33
From market. Variety un-	Raw	0.32 [1]		
known	Roasted 1 hour and 55 min. with beef			
	Leaves separated		0.11 [1]	34
Rutabaga. From market				
Variety unknown. Radial sectors of cross sections of 3 rutabagas	Raw	0.44 [1]		
Approx. 1 in. strips, ¾ in. thick	Boiled 25 min. Small amount of water		0.38 [1]	86
Approx. 2 in. squares, ½ in. thick	Boiled 1½ hours		0.22 [1]	50
Fiddlehead greens (Onoclea struthiopteris)	Raw	0.45		
Native wild greens, gather- ed locally	Boiled 30 min. Small amount of water		0.32	71
Sample—14 heads	Boiled 2 hours		0.20	44

[1] Dehydro ascorbic acid not determined. Figures represent reduced values.

of cabbages were grown at Orono, Maine during the summer of 1939.

The values for mature cabbages consist of an average of from three to five samples of each variety covering a period of from 6 to 10 weeks. At maturity, the average amounts of reduced ascorbic acid in cabbage types were as follows:

TYPE	VARIETIES	MG. PER GM.
Ballhead	3	0.48
Early Savoy	3	0.46
Miscellaneous. Late	2	0.43
Early Wakefield	2	0.43
Midseason	2	0.42
Early Copenhagen	2	0.36

Losses sustained during cooking were investigated. Of ninety-three housewives interviewed, only seventy reported cooking techniques for cabbages. The lengths of the cooking periods reported most frequently were 30, 60 and 120 minutes. Complete analytic data are recorded in table 1.

Rutabaga. Rutabaga was found to be a relatively potent source of ascorbic acid. To it may be ascribed all of the advantages enumerated for cabbage, viz., facility of production and storage and familiarity to the consumer. This vegetable was reported by fourteen of ninety-one children in the spring and by eighteen of seventy-six children in the autumn. The amounts of ascorbic acid existing in nine varieties of Maine-grown rutabaga ranged from 0.44 mg. per gram for the Macomber variety to 0.61 mg. per gram for the Large White French variety (McIntosh, '38), thus making the choice or variety to be produced and/or consumed an important one.

Data resulting from the analyses of rutabaga are recorded in table 1.

Greens. Greens were reported by twelve of ninety-one children in the spring and eleven of seventy-six children in the fall. Most of the greens reported consisted of the home-canned or home-salted types which furnish very little ascorbic acid as shown in table 2. Fiddlehead greens, a wild

native green which is a very popular dietary item during May and June, were chosen as typical greens to demonstrate losses incurred during cooking according to the methods in use. Cooking losses are shown in table 1.

Tomatoes. In the spring the ninety-one children reported eating tomato products (raw, canned, or juice) seventy-eight times and in the fall seventy-six children reported them fifty-five times. These products were obtained on the market. The most popular brands were analyzed and the data are recorded in table 2.

TABLE 2

Ascorbic acid content of canned foods reported on dietaries

MATERIAL	METHOD OF PREPARATION	TOTAL ASCORBIC ACID MG. PER GM. AS TAKEN FROM JARS
Home-salted greens: spinach, beet, and turnip tops	Raw greens and dry salt	0
Home-canned fiddle-head greens	No record. Obviously cooked.	0.04 [1]
Home-canned turnip greens	Cooked 2½ hours	0.07 [2]
Home-canned cultivated strawberries	1 part sugar to 1 part berries. Simmer 6 min. Set in sun for 4 days. Pack	0.06
Home-canned field strawberries	1 part sugar to 1 part berries. Stand overnight. Warm on back of stove. Pack	0.04
Home-canned field strawberries	1 part sugar to 1 part berries. Stand 24 hours. Seal in hot jars	0.04
Home-canned raw rhubarb	Rhubarb and cold water	0.06
Home-canned rhubarb sauce	Raw and sugar. Heated 10–15 min.	0.06
Tomato juice, market	Brand A	0.15
Canned tomatoes, market	Brand B	0.12
Canned tomatoes, market	Brand C	0.11

[1] Fifty per cent loss when heated to serve.
[2] Forty-three per cent loss when heated to serve.

Undoubtedly during the growing season fresh tomatoes furnish a more substantial part of the vitamin C supply as they are produced to a small extent in the region. The brevity of the growing season necessitates an early variety. A typical variety grown in the locality is Penn State Earliana. This

variety was produced and analyzed at Orono, Maine during the summers of 1938 and 1939. Of fifteen samples tested, the values ranged from 0.17 to 0.25 with a mean of 0.21 mg. per gram for ripe fresh tomatoes. This was the second lowest value obtained of twenty-four varieties tested. The mean values for all of the tomato varieties analyzed ranged from 0.17 mg. per gram to 0.33 mg. per gram. An early high vitamin C variety of tomato adapted to the soil and climate would be a desirable substitute for the Earliana.

Apples. In the spring, thirty-eight of ninety-one children and forty of seventy-six in the autumn reported the consumption of raw apples. It was not possible to determine the variety which was most frequently eaten. Determinations have shown that certain varieties of apples contain five times as much vitamin C as do others (Murphy, '39). In view of these large varietal differences the exchange of one variety for another would deprive or assure Maine people of part of their yearly vitamin C supply (Dove and Murphy, '36).

Miscellaneous. Other vitamin C containing foods occurring in the dietaries were found not to contain very large amounts of this food essential. Canned field strawberries and canned rhubarb were analyzed and the resulting data are tabulated in table 2.

DISCUSSION

On the whole, analyses of the food records and determinations of the vitamin C values of the foods involved, demonstrate the inadequacy of this factor in the dietaries.

The above figures tend to show that potatoes, especially in the fall, may provide a valuable part of the daily vitamin C requirement. Smith ('38) has tentatively suggested that 50 mg. daily would supply the usual needs of an adult with but little margin of safety. If potatoes are the sole source of vitamin C, to provide a daily intake of 50 mg. would necessitate eating seven average-sized (5⅓ ounces) peeled, boiled potatoes per day in the spring. This would represent a calorie intake of slightly less than 900 calories. In the autumn three and one-

half boiled potatoes daily would provide a similar amount of ascorbic acid, representing somewhat less than 500 calories.

Over two-thirds of the children were eating potatoes about once a day and were obtaining from this source about 15 mg. of ascorbic acid daily in the fall and 8 mg. daily in the spring. These amounts, although not considered adequate for optimum health, were perhaps large enough to prevent the manifestation of the usually recognized symptoms of clinical scurvy. On the other hand, the low plasma values, as well as the prevalence of gum inflammation, indicate that potatoes were not supplying enough vitamin C to make the inclusion of other vegetables and fruits in the diet unimportant.

In native-grown and home-stored cabbage lies a potential supply of vitamin C in amounts large enough to furnish adequately man's requirements without over-emphasizing the place of cabbage in the diet. More frequent consumption of cabbage in the raw state would enhance the nutritional value received. Losses incurred during the cooking process are large but even after 2 hours of boiling, 23 mg. per 100 gm. of vitamin C remain. By using shorter cooking times and diminishing the amount of cooking water, the vitamin C remaining available in cabbages ranged from 31 to 38 mg. per 100 gm. Vitamin C needs could be supplied with an intake of less than 100 calories from cooked cabbages.

Rutabaga also is a locally produced and stored vegetable which could provide a very substantial portion of vitamin C needs.

Fresh greens in the raw state are reported by Burrell and Miller ('39), Fenton, Tressler, Camp and King ('37), and Tressler, Mack and King ('36) to contain relatively large amounts of ascorbic acid. Greens cooked for long periods of time and in large amounts of water cannot be depended upon to contribute materially to vitamin C requirements. The home-canned greens were found to be of little value and salted greens of no value in furnishing this food essential during the winter period of shortage.

Raw and canned tomatoes could easily be utilized to good advantage in providing vitamin C. In this respect it may be well to point out that certain varieties of tomatoes furnish twice as much of the vitamin for a given weight of tomato as do other varieties. If the original content is high, obviously the amount remaining after processing would be of more value to the consumer. Therefore, nutritional superiority, as well as adaptability to soil and climate, should be considered an important criterion in evaluating varieties of fruits and vegetables to be produced for home or market consumption.

The same qualifications may be applied when considering apples as a source of vitamin C. The choice of variety is all-important if the consumer depends on apples for part of his daily supply.

SUMMARY

A study of vitamin C nutrition, as judged by plasma ascorbic acid values and by oral conditions reported elsewhere has been amplified in this report by a study of the diet records and by determinations of total ascorbic acid in the foods consumed by the children under observation.

Analyses of seventy-six fall and sixty-three spring diet records showed that only one child in seven attained the minimum dietary standard of one good vitamin C food daily.

Storage losses were demonstrated in potatoes, the chief source of vitamin C in the dietaries observed. The ascorbic acid values of raw Green Mountain potatoes decreased from 0.17 mg. per gram in the fall to 0.11 mg. per gram in the spring.

Losses during cooking, according to the techniques employed in the region, were as follows: for potatoes, 27 to 55%; for cabbages, 46 to 67%; for rutabagas, 14 to 50%; and for fiddlehead greens, 29 to 56%.

The findings of vitamin C undernutrition in which 45 and 63% of the children examined showed plasma ascorbic acid values of less than 0.40 mg. per cent, while 32 and 51% of

the children showed oral inflammation, are here shown to be paralleled by markedly inadequate vitamin C intakes in 58 and 40% of the dietaries examined. The large losses of vitamin C attributed to the effects of storage and poor cooking methods emphasize the dietary relation to the clinical symptoms.

ACKNOWLEDGMENTS

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THE EFFECT OF CALCIUM AND PHOSPHORUS ON THE METABOLISM OF LEAD

J. B. SHIELDS AND H. H. MITCHELL

Division of Animal Nutrition, University of Illinois, Urbana

The greater part of the lead hazard to which the average American is exposed and which Minot ('38) has recently assessed, is contributed by the food, and of the intake of lead in the food, an undetermined but probably not inconsiderable fraction is contributed by fruits that have been sprayed with lead arsenate for the control of insect pests during the development of the fruit. The fact that the Federal tolerance for lead has been raised in the last 2 years from 0.019 to 0.050 grain per pound of food affords some basis for the belief that the lead intake of the average American will increase in proportion to the consumption of fruit sprayed with lead arsenate. The fact that these usual and presumably sub-toxic levels of lead consumption seem to induce a slow but significant accumulation of lead in the human body (Morris, '40), should not be lost sight of in this connection.

Since modern life inevitably involves a lead hazard, it is important to explore the possibility of minimizing its effects on the body. The intake of lead, like the intake of calcium, is not a good measure of the physiological effect to be expected, since the body assimilates dietary components to different degrees of completeness depending among other things upon the direct effect of one dietary component on the assimilation of another. As an instance of such a relationship may be mentioned the marked effect of some apple constituent or constituents in depressing the assimilation of lead (Shields, Mitchell and Ruth, '39 b).

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The element calcium seems to possess to a preëminent degree the property of disturbing the assimilation of other minerals. Its relation to phosphorus assimilation is well known. The utilization of phytic acid in particular is impaired by calcium to phosphorus ratios of 2 to 1 or higher (Krieger and Steenbock, '40). Many salts of calcium greatly reduce the assimilation of iron (Kletzien, Buchwald and Hudson, '40), while high ratios of calcium to phosphorus produce the same effect (Anderson, McDonough and Elvehjem, '40). Excess calcium in the diet of chicks depresses the assimilation of manganese (Wilgus and Patton, '39), this effect resulting apparently from interactions between the two elements in the digestive tract (Caskey and Norris, '39). In a similar manner, an excess of calcium will impair the assimilation of iodine (Thompson, '36). However, these effects of excess dietary calcium are not all detrimental to the animal, since a high level of calcium in the diet may counteract the deleterious effects of high levels of iodine (Thompson, '36) and of manganese (Becker and McCollum, '38).

The retention of lead in the body and its detrimental effects on physiological processes may also be counteracted by excess dietary calcium. In fact, the triumvirate of factors controlling calcification of the bones, dietary calcium, phosphorus and vitamin D, have been claimed to modify the retention of lead. Sobel, Yuska, Peters and Kramer ('40) have shown that the addition of either calcium or phosphorus to a low-calcium low-phosphorus diet containing added lead will depress lead retention in rats, while the addition of vitamin D to any of the types of diets studied by them will enhance lead retention. Shelling ('32–'33) and Shelling and Hopper ('36) emphasize in particular the effect of phosphorus in the control of lead toxicity. Calvery and his associates (Grant, Calvery, Laug and Morris, '38), also, found with rats that lead retention was decreased by increasing the calcium content of the diet, but at the same time the phosphorus content of the diet was lowered. The experiments of Tompsett ('39) and of Tompsett and Chalmers ('39) on adult mice confirm the depressing effect

of high-calcium diets on lead retention. That the effect of calcium on the assimilation of lead is dependent mainly or entirely upon events occurring in the digestive tract, is shown by the experiments of Lederer and Bing ('40). In these experiments, the retention of lead injected intraperitoneally into rats was independent of the calcium and phosphorus contents of the diet, while lead incorporated in the diet was retained in much greater amount in bone and kidney, but not in liver, when the calcium content of the diet was low than when it was high.

Most of the experiments on the effect of calcium, phosphorus and vitamin D on the metabolism of lead involved the use of diets containing much higher concentrations of lead than would be of significance in practical nutrition. Shelling used rations containing 1.2% of lead; Sobel used rations containing 0.80 to 0.82% of lead, with much higher concentrations in his earlier work; Tompsett in most of his experiments fed 1 mg. of lead to each mouse daily, equivalent to 0.04% of the ration; Lederer and Bing fed diets containing 100 p.p.m. of lead. Calvery and his associates ('38), in their studies of similar import, fed diets varying in lead content from 13 to 512 p.p.m.

It seemed important to re-explore the field of the relationships between dietary calcium, phosphorus and lead from the standpoint of the lead hazard existing in the American diet. Are the relationships the same for low concentrations of dietary lead as for the high concentrations usually studied in the past? The present Federal tolerance for lead is equivalent only to 7.1 p.p.m. of fresh fruit, or about 42 p.p.m. of dried fruit (apple).

PLAN OF EXPERIMENTS

The experiments were planned to compare the retentions of lead in growing and adult rats induced by diets containing a constant proportion of lead, but variable proportions of calcium with phosphorus constant, of phosphorus with calcium constant, and of both calcium and phosphorus, the variations being simultaneous. The lead concentration of the diets varied

from about 15 to 33 p.p.m. of dry matter. The lead was added in all cases in the form of acid arsenate, PbHAsO$_4$, commonly used as a spray chemical.

The composition of the basal diets used in the five experiments to be reported in this paper is given in table 1. To these basal diets, supplements of PbHAsO$_4$, CaHPO$_4$, Na$_2$HPO$_4$ and CaSO$_4$ were added to give the desired concentrations of lead, calcium and phosphorus.

TABLE 1

Description of experimental basal diets, expressed in per cent of ingredients

INGREDIENTS	BASAL DIETS USED IN EXPERIMENTS NUMBER			
	184 and 185	229	165	212
Dried and extracted beef	12.5	0	0	12.0
Dried and extracted egg	12.5	0	23.0	0
Egg albumin	0	20.0	0	0
Butter fat	4.0	0	6.0	0
Lard	8.0	0	9.0	12.0
Corn oil	0	12.0	0	0
Sucrose	10.0	10.0	10.0	10.0
Starch	32.5	41.5	24.5	48.5
Agar	1.0	1.0	1.0	1.0
Cod liver oil	1.0	1.0	1.0	1.0
Wheat germ oil	0.5	0.5	0.5	0.5
Dried yeast	10.0	6.0	10.0	10.0
Salt mixture (Ca- and P-free)[1]	8.0	8.0	15.0	5.0
Total	100.0	100.0	100.0	100.0

[1] Contains from 50 to 67% starch to facilitate mixing.

The rats were fed in groups of two, three or four, depending upon the number of rations to be compared, and within each group the food intakes were equalized. Each group of rats was of the same sex, and generally of the same litter, and were selected to be approximately equal in body weight. Representative rats from each litter contributing rats to the experiment were sacrificed and analyzed for lead, and for calcium and phosphorus when retentions of these elements during the experimental feeding periods were estimated.

This equalization of the food intakes of grouped rats on comparable rations is considered to be an essential feature

of the experiments, since only under these conditions will the intakes of lead, calcium and phosphorus among the rations to be compared be in the desired proportions, and only under these conditions will the intake of other nutrients be the same. Hence, only under conditions of equal intakes of food can the results be interpreted strictly in terms of the differences in composition of the experimental rations.

At the termination of the feeding periods, lasting generally from 7 to 22 weeks, the rats were killed, the body length from tip of nose to root of tail measured, and the empty carcass of each analyzed for lead and occasionally for calcium and phosphorus, either in the whole carcass or in bones and soft tissues, separated after autoclaving. Each group of rats was sacrificed when it had consumed a definite amount of food, the same for each group in its respective experiment. The amounts of food consumed were generally 500 gm., 600 gm., or 1000 gm. per rat.

The method of lead analysis used is a modification of the dithizone method, described in a previous publication from this laboratory (Shields, Mitchell and Ruth, '39 a). The calcium analyses were carried out according to the method of McCrudden ('11–'12) and the phosphorus analyses according to the official method of the A.O.A.C. for phosphorus in fertilizer.

DISCUSSION OF EXPERIMENT RESULTS

The average results of the lead metabolism studies are summarized in table 2; the average results on the distribution of the retained lead between bone and soft tissues, insofar as this was determined, will be found in table 3, while the limited amount of data on calcium and phosphorus retention and on the content of these elements in the experimental carcasses is assembled in table 4. Table 5 contains statistical analyses according to the method of Student ('08; '25) of the significant comparisons of the average data reported in the preceding three tables.

The results of experiments 229 and 185 (table 2) show that diets containing 0.12 and 0.38% of phosphorus, and calcium

TABLE 2

Lead metabolism data. Averages per rat

EXPT. NO.	COMPOSITION OF DIETS: Calcium	Phosphorus	Lead	NUMBER OF RATS IN GROUP	DAYS ON TEST	TOTAL FOOD CONSUMED	INITIAL BODY WEIGHT	TOTAL GAIN IN BODY WEIGHT	ATTAINED BODY LENGTH	TOTAL LEAD IN CARCASS	ESTIMATED LEAD RETENTION Total	Per cent of intake
	%	%	p.p.m.			gm.	gm.	gm.	mm.	mg.	mg.	
185	0.55	0.38	15.8	6	76	600	42	134	204	0.380	0.269	2.8
	0.53	0.68	15.3	6	76	600	41	136	204	0.215	0.104	1.1
	0.54	1.14	15.7	6	76	600	41	117	201	0.252	0.141	1.5
229	0.19	0.12	23.7	11	73	500	45	98	184	0.868	0.823	6.9
	0.19	0.43	23.1	11	73	500	45	97	184	0.601	0.557	4.8
	0.18	0.82	27.3	11	73	500	41	89	183	0.607	0.562	4.1
184	0.27	0.53	16.6	4	73	600	37	162	209	0.339	0.231	2.8
	0.54	0.53	16.8	4	73	600	36	148	206	0.197	0.089	0.9
	1.11	0.54	16.6	4	73	600	36	147	208	0.231	0.123	1.2
165	0.10	0.37	16.9	11	156	1001	52	148	216	2.180	2.122	12.5
	0.65	0.61	16.7	11	156	1001	51	155	216	1.019	0.961	5.7
	1.08	1.06	17.2	11	156	1001	52	154	217	0.484	0.426	2.4
212	0.03	0.27	2.1	8	48	481	395	− 9	—	0.272	0	—
	0.03	0.26	32.6	8	47	475	392	− 27	—	1.360	1.088 [1]	7.6 [2]
	0.12	0.32	32.1	8	48	481	393	− 12	—	1.174	0.903 [1]	6.2 [2]
	0.46	0.34	29.8	8	48	481	397	− 13	—	0.447	0.175 [1]	1.3 [2]

[1] Lead retentions above that of rats on basal diet containing no added lead.
[2] Based on lead intake above that of rats on basal diet containing no added lead.

to phosphorus ratios of 1.4:1 and 1.6:1, respectively, definitely promote retention of the contained lead, as compared with diets containing larger proportions of phosphorus but no more calcium. However, concentrations of phosphorus greater than 0.43% in experiment 229 and 0.68% in experiment 185, did not depress lead storage further. In fact, there was a

TABLE 3

Distribution of lead stores in body. Averages per rat

EXPT. NO.	COMPOSITION OF DIETS:			NUMBER OF RATS IN GROUP	LEAD CONTENT:		PER CENT OF TOTAL BODY Pb IN SOFT TISSUES
	Calcium	Phosphorus	Lead		In soft tissues	In bones	
	%	%	p.p.m.		mg.	mg.	
165	0.10	0.37	16.9	6	0.246	1.875	11.62
	0.65	0.61	16.7	6	0.156	0.875	15.11
	1.08	1.06	17.2	6	0.087	0.421	17.25
212	0.03	0.27	2.1	5	0.082	0.189	31.2
	0.03	0.26	32.6	5	0.195	1.112	15.0
	0.12	0.32	32.1	4	0.197	0.989	19.2
	0.46	0.34	29.8	5	0.149	0.382	24.8

TABLE 4

Calcium and phosphorus metabolism data. Averages per rat

EXPT. NO.	COMPOSITION OF DIETS		CALCIUM IN CARCASS		ESTIMATED CALCIUM RETENTION	PHOSPHORUS IN CARCASS		ESTIMATED PHOSPHORUS RETENTION
	Calcium	Phosphorus						
	%	%	gm.	%	gm.	gm.	%	gm.
229	0.19	0.12	1.166	0.82	0.748	0.869	0.614	0.567
	0.19	0.43	1.194	0.85	0.774	0.881	0.623	0.577
	0.18	0.82	1.140	0.86	0.724	0.838	0.628	0.537
165	0.10	0.37	1.592[1]	0.83	—	1.182	0.60	—
	0.65	0.61	2.009[1]	0.98	—	1.307	0.64	—
	1.08	1.06	2.097[1]	1.04	—	1.386	0.69	—

[1] In bones or carcass.

tendency at the higher phosphorus levels, though it was not significant statistically, for the lead retentions to be somewhat greater than at the intermediate levels.

The decrease in lead retention from the low to the medium phosphorus level in experiment 229, was not associated with an appreciable or significant increase in the retention of either

TABLE 5

Statistical analysis of results reported in tables 2, 3, and 4

EXPT. NO.	RATIONS COMPARED	DESCRIPTION OF EXPERIMENTAL DATA	STATISTICAL RESULTS:			NUMBER OF PAIRED DIFFERENCES
			Average difference	Standard deviation	Probability [1]	
185	Low P — medium P	Pb retention, mg.	0.165	0.115	0.012	6
	Low P — high P	" " "	0.128	0.113	0.026	6
	High P — medium P	" " "	0.037	0.091	0.20	6
229	Low P — medium P	Pb retention, mg.	0.266	0.284	0.007	11
	Low P — high P	" " "	0.261	0.374	0.026	11
	High P — medium P	" " "	0.006	0.218	0.47	11
	Medium P — low P	Ca retention, gm.	0.0260	0.0478	0.058	11
	Medium P — high P	" " "	0.0501	0.0563	0.009	11
	Low P — high P	" " "	0.0241	0.0377	0.035	11
	Medium P — low P	P retention, gm.	0.0100	0.0285	0.15	11
	Medium P — high P	" " "	0.0401	0.0334	0.0017	11
184	Low Ca — medium Ca	Pb retention, mg.	0.142	0.069	0.021	4
	Low Ca — high Ca	" " "	0.108	0.091	0.067	4
	High Ca — medium Ca	" " "	0.034	0.057	0.10	4
165	Low (Ca + P) — medium (Ca + P)	Pb retention, mg.	1.161	0.253	<0.00001	11
	Low (Ca + P) — high (Ca + P)	" " "	1.696	0.270	<0.00001	11
	Medium (Ca + P) — high (Ca + P)	" " "	0.535	0.145	<0.00001	11
	Low (Ca + P) — medium (Ca + P)	Pb in soft tissues, mg.	0.090	0.041	0.0023	6
	Medium (Ca + P) — high (Ca + P)	" " "	0.069	0.033	0.0027	6
	Medium (Ca + P) — low (Ca + P)	P in carcass, gm.	0.125	0.080	0.0003	11
	High (Ca + P) — medium (Ca + P)	" " "	0.079	0.099	0.014	11
	Medium (Ca + P) — low (Ca + P)	Ca in carcass, gm.	0.649	0.446	0.022	5
	High (Ca + P) — medium (Ca + P)	" " "	0.089	0.328	0.31	5
	Medium (Ca + P) — low (Ca + P)	Ca in bones, gm.	0.223	0.135	0.0072	6
	High (Ca + P) — medium (Ca + P)	" " "	0.088	0.073	0.021	6
212	Low Ca — medium Ca	Pb retention, mg.	0.185	0.344	0.10	8
	Medium Ca — high Ca	" " "	0.728	0.395	0.0009	8
	High (Ca + Pb) — low Ca (no added Pb)	" " "	0.175	0.175	0.017	8
	Low Ca — high Ca	Pb in soft tissues, mg.	0.046	0.030	0.019	5
	Medium Ca — high Ca	" " "	0.057	0.051	0.074	4

[1] Probability to fortuitous factors alone would bring about an average difference as great as, or greater than, the observed average difference.

calcium or phosphorus (table 4). The retention of both calcium and phosphorus was definitely depressed when the phosphorus level was raised to 0.82%, with a calcium to phosphorus ratio of 0.22 : 1. Evidently in this experiment there was no tendency for lead deposition to parallel calcium or phosphorus deposition. On the contrary, the relationship tended to be an inverse one.

Experiment 184 permits a study on growing rats of the effect on lead deposition of increasing levels of calcium, the phosphorus level remaining constant. As the calcium content of the diet increased from 0.27 to 0.54%, the retention of lead decreased to less than half. A further increase in the calcium level to 1.11 and in the Ca : P ratio from 1 : 1 to approximately 2 : 1, produced an inappreciable and statistically insignificant increase in the deposition of lead.

Experiment 212 permits the same sort of a comparison with mature rats. Here an increase in the level of calcium in the diet from 0.03 or 0.12 to 0.46% produced a marked and significant reduction in lead storage, both in the entire carcass, in the bones and in the soft tissues (table 3). The reduction in the lead content of the soft tissues by an increase in the calcium content of the diet to only a moderate level, seems especially significant. With the same lead content in a diet containing 1.24% of calcium, a previous experiment (Shields, Mitchell and Ruth, '39 a) had failed to demonstrate any retention of lead in mature rats.

From table 3 it may also be seen that the proportion of the total content of lead in the carcass that is present in the soft tissues varies inversely as the total content. The results of experiment 165 also support this statement. For low contents of lead, 25 to 30% may be present in the soft tissues.

In experiment 165, the effect on the retention of lead in young rats of increasing simultaneously the proportion of dietary calcium and phosphorus was tested. Here the lead retention and the lead content of the carcass, bones and soft tissues decreased greatly and significantly with the increasing mineral content of the diet. On the contrary the calcium and phosphorus contents of the carcass and the calcium content of

the bones increased with the increase in the concentration of these elements in the diet, especially in the comparison of the low-mineral and medium-mineral diet. Here again the retention of calcium responds to a change in the calcium content of the diet in a way diametrically opposed to that of the retention of lead.

The results of these experiments do not appear to conform with the theory of Sobel, that the factors controlling the deposition of lead in the bones are similar to those controlling the assimilation of calcium and in particular that there is a most favorable ratio of lead to phosphorus of approximately 3 to 1. Nor do they conform with the theory of Shelling, which emphasizes the importance in lead assimilation of the presence of available phosphorus in the diet. Furthermore, they demonstrate that the lead stream from the intestinal tract to the tissues may not vary with the calcium stream.

There would seem to be at least two spheres of reaction within which the effects of dietary calcium and dietary phosphorus on lead assimilation are mediated. One sphere is the lumen of the gastrointestinal tract, where the absorption of dietary lead appears to be impaired by concentrations of calcium or phosphorus or both above certain low limits. The other sphere is the area of mineral interchange between blood and bone trabeculae. The less active this interchange becomes, due either to advancing age or to the saturation of mineral stores, the less opportunity is there for an active deposition of any bone mineral, including lead.

SUMMARY AND CONCLUSIONS

The effect of varying the concentration of calcium and phosphorus in diets containing low concentrations of lead (16 to 32 p.p.m.) on the retention of lead was investigated in a series of five experiments involving 128 growing or adult rats. In some of these experiments the retention of calcium and phosphorus was also measured. All mineral retentions were measured by carcass analysis. An essential feature of the experiments was the equalization of the food intakes of rats on comparable diets, thus permitting the interpretation

of the results in terms solely of the relative compositions of the diets.

The results obtained appear to warrant the following conclusions:

1. A low content of calcium or of phosphorus or of both in the diet induces a high retention of lead in comparison with diets containing higher mineral levels. In fact, the only method of securing lead storage in adult rats on the moderate concentration of dietary lead used (32 p.p.m.) is to lower the calcium content of the diet to inadequate or borderline levels.

2. Excessive dietary levels of calcium and phosphorus are not appreciably more protective against the assimilation of lead by the body than are levels approximating the requirements. This statement applies only to the moderate levels of dietary lead employed.

3. Under the imposed conditions of variable dietary concentrations of calcium and phosphorus, the retention of calcium runs in a diametrically opposite direction to the retention of lead.

4. Under conditions of practical nutrition, an adequate intake of calcium and of phosphorus presumably protects the body against appreciable assimilation of the low levels of dietary lead involved in the usual lead hazard of modern life. This protection is more effective in the adult than in the adolescent for any given concentration of calcium and phosphorus, possibly because the mineral metabolism of the bone trabeculae is considerably less intense in the adult than in the growing organisms.

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RESPIRATORY METABOLISM IN FRUCTOSURIA [1]

HENDERIKA J. RYNBERGEN, WILLIAM H. CHAMBERS AND
N. R. BLATHERWICK

*Department of Physiology, Cornell University Medical College, and the
Biochemical Laboratory, Metropolitan Life Insurance Company, New York City*

ONE FIGURE

INTRODUCTION

Fructosuria is a disturbance of metabolism in which part of the fructose ingested is excreted in the urine. It may occur uncomplicated by other disturbances of carbohydrate metabolism, or it may appear in combination with the excretion of glucose. This paper will deal with the former condition, essential fructosuria.

The first case of probable fructosuria presented in the literature is that of Zimmer and Czapek in 1876. Including this case, Silver and Reiner, reporting on three additional patients, list a total of twenty-seven cases of fructosuria up to 1934. Recent observations besides those of Silver and Reiner ('34) have been reported by Heeres and Vos ('29), Marble and Smith ('36), van Lookeren Campagne ('36), and Edhem, Erden and Steinitz ('38). These investigators describe the disorder as characterized by the excretion of a constant proportion of ingested fructose, amounting to between 10 and 20%, and an elevation of the total blood sugar level due to the presence of fructose despite a small depression in blood glucose.

In 1935 Blatherwick (unpublished data) noted the absence of any appreciable rise in blood lactate following fructose ingestion in two individuals with fructosuria (subject A. O.

of this paper and his sister). Recently Edhem, Erden and
Steinitz ('38) found similar results with respect to blood
lactic acid in their subject. The only respiratory metabolism
data are those of Heeres and Vos who report that their
subject showed a rise in quotient to 0.77 from a basal level of
0.72 one hour after fructose ingestion, compared with a rise
from the basal quotient of 0.71 to a quotient of 0.95 one hour
after fructose ingestion in a normal control. Although data
beyond the first hour after fructose ingestion are not given, the
authors conclude that no combustion of sugar takes place.
Therefore a more complete study of the respiratory metabo-
lism and its relation to the lactic acid changes in the blood was
undertaken.

EXPERIMENTAL DATA

Of the four available subjects, only two were sufficiently
cooperative for reliable respiratory metabolism data. We are
indebted to Drs. B. S. Oppenheimer and S. Silver for referring
subject A. R., who is case 1 in the report of Silver and Reiner.
He is of Italian parentage, male, 29 years old, 157 cm. tall,
and weighs 53 kg. Subject A. O. is a deaf-mute of Hungarian
extraction, male, age 25 years, height 167 cm., and weighs 77
kg. A younger brother of A. R., as well as the sister of A. O.,
also show the disorder.

Respiratory metabolism was determined in approximately
30-minute periods with the Benedict Universal apparatus.
The data presented in table 1 have been calculated on the basis
of 30-minute periods, disregarding the necessary 6- to 10-
minute intervals between periods. The subjects came to the
laboratory in the post-absorptive state. After resting for
about an hour the basal metabolism level was established and,
except where otherwise noted in table 1, 50 gm. of sugar in
250 cc. of water at 37°C. were given orally. The metabolism
determinations were continued until the subject returned to
approximately the basal level.

Urines were collected for the basal periods and after each
sugar experiment, according to the time intervals shown in

TABLE 1

Changes in non-protein respiratory quotients and heat production following ingestion of sugar

DATE	INGESTED		30-MINUTE PERIODS	BASAL METABOLISM					AFTER INGESTION OF SUGAR								
	Sugar	H₂O		1	2	3	4	Av.	1	2	3	4	5	6	7	8	9
	gm.	*cc.*															
								Subject A. R.									
7/17	50 gluc.	250	R.Q.	0.78	0.78	0.75	0.74	0.76	0.72	0.83	0.83	0.90	0.87				
			Cals.	28.0	27.5	28.2	28.7	28.1	30.2	30.3	28.5	31.4	27.9				
7/24	50 fruct.	250	R.Q.	0.75	0.79	0.74		0.76	0.82	0.87	0.87	0.91	0.82	0.72	0.72		
			Cals.	27.7	27.6	28.6		28.0	27.6	28.7	30.0	29.2	30.7	28.3	28.5		
7/31[1]	50 fruct.	500	R.Q.	0.73	0.71	0.71	0.70	0.71	0.73	0.73	0.81	0.83	0.78	0.74	0.73	0.72	
			Cals.	27.7	27.7	28.0	27.7	27.8	29.5	30.2	30.6	31.1	30.1	29.8	28.9	29.3	
								Subject A. O.									
3/13	50 fruct.	250	R.Q.	0.80	0.84	0.90	0.84	0.85	1.04	0.82	0.85	0.83	0.84				
			Cals.	36.7	35.6	34.5	33.9	35.2	35.3	37.2	35.6	36.2	35.9				
3/20	50 fruct.	250	R.Q.	0.88	0.84	0.77		0.83	0.81	0.82	0.84	0.83	0.85				
			Cals.	34.3	34.9	35.2		34.8	34.6	36.5	36.2	37.0	36.6				
4/3	50 + 50 fruct.	250 + 250	R.Q.	0.88	0.83	0.94		0.85	0.89	0.86	0.87	0.87	0.84↑	0.93	0.93	0.88	0.88
			Cals.	34.4	35.5	35.9		35.2	35.3	35.7	36.9	37.1	35.5↓	36.4	36.4	36.5	36.4
4/10	50 + 50 fruct.	250 + 250	R.Q.	0.92	0.90	0.86	0.89	0.89	0.88	0.85	0.91	0.89↑	0.95	0.91	0.83		
			Cals.	32.7	33.4	33.5	34.4	33.5	34.8	35.1	36.3	36.3↓	35.8	36.4	37.0		
6/26[2]	50 + 50 fruct.	250 + 250	R.Q.	0.88	0.86	0.80		0.84	0.92	0.89	0.91	0.91↑	0.92	0.90	0.81	0.83	
			Cals.	35.2	36.2	36.0		35.8	35.8	36.6	37.0	36.6↓	37.8	37.9	36.6	39.1	

↑↓ Second dose of fructose.

[1] Subject on a low carbohydrate intake for 2 days previous to this experiment.

[2] Bloods taken for fructose determination during this experiment.

table 3. Nitrogen was determined by the Kjeldahl method and fructose according to Roe ('34). The latter agreed within 0.3% with the total sugar by the Shaffer-Somogyi method ('33), and by polariscope, after clarifying the urine with neutral lead acetate.

TABLE 2

Lactic acid and sugar concentration in blood following fructose ingestion

SUBJECT	DATE	VENOUS BLOOD	HOURS AFTER FRUCTOSE INGESTION						
			0	0.5	1	2	3	4	6
			mg.%	mg.%	mg.%	mg.%	mg.%	mg.%	mg.%
A.R.	8/7/37	Lactic acid	9.0	7.1	10.8	11.4		12.2	
		Fructose	0	40	48	54		12	
		Glucose	93	87	91	86		84	
		Total sugar	93	125	137	138		95	
A.R.	1/8/38	Lactic acid	9.0	7.7	7.0	8.2		10.1	10.1
		Fructose	0	29	45	42		17	Trace
		Glucose	82	73	70	68		73	80
		Total sugar	82	101	113	109		89	80
A.O.	4/17/35	Lactic acid	10.8	13.8		10.9	10.1		
		Fructose	0	40		43	22		
		Glucose	86	76		81	83		
		Total sugar	86	116		124	105		
A.O.	6/26/37	Lactic acid	7.1	7.2			13.5	13.3 [1]	
		Fructose	Trace	23			36	47	
		Glucose	78	76			74	74	
		Total sugar	78	98			108	119	

[1] Forty minutes after second dose of fructose.

The blood fructose and lactate experiments were conducted on different days from the respiratory metabolism experiments except the experiment on A. O. of June 26th. The same precautions as to basal conditions and administration of sugar were observed. Proteins were precipitated from 5-cc. samples of venous blood by the Somogyi ('30) procedure, and the total blood sugar determined according to Shaffer and Somogyi ('33), the fructose by Roe's method, and the lactate by the method of Friedemann and Graeser ('33) as modified by Blatherwick et al. ('35). Chemically pure sugars were administered. This is essential in the case of fructose to prevent the diarrhoea that is occasioned by its impurities (Rowe, Plummer and McManus, '33).

Control experiments were carried out on three normal subjects. Since the results agree with those established by other investigators (Deuel, '36), the data are not included in this paper.

The metabolism studies on the two fructosuric subjects are shown in table 1. The first observations on subject A. R. (July 17th) were a control test with 50 gm. of glucose. The reaction was similar to that in the normal individual, a slow rise in non-protein respiratory quotient which reached a peak at 0.90 in the fourth period, about 2½ hours after ingestion of the sugar (Cathcart and Markowitz, '27; Carpenter and Fox, '30 a). The respiratory quotients after 50 gm. of fructose (July 24th) followed the same curve to a high point of 0.91 in the fourth period, thus indicating an oxidation of fructose similar to that of glucose in this individual. In order to reduce the carbohydrate metabolism a diet low in carbohydrate, limited to 50 gm. of lactose in milk, was taken for 2 days prior to the fructose experiment of July 31st. The basal R.Q. was low, 0.71, and ketones were present in both basal and post-fructose urines. The rise in respiratory quotient after fructose ingestion followed the same curve as previously, but at a lower level, with the highest quotient at 0.83, thus agreeing with glucose studies on normal individuals under similar dietary conditions (Chambers, '38). These results suggest a transformation of fructose into glucose with subsequent oxidation of the latter sugar.

The basal metabolism of subject A. R. remained at the same level during the three experiments (table 1), averaging 37.3 calories per square meter per hour or —8 according to the DuBois standard. Following the ingestion of fructose the rise in heat production was similar to that after glucose was taken, reaching in the fourth or fifth period a maximum specific dynamic action of about 6 calories per hour, which is within the limits found in the normal individual (Carpenter and Fox, '30 b).

The results with the other subject (A. O.) differ from those just cited. The non-protein respiratory quotients for the

basal periods were consistently higher, averaging 0.85, although the individual periods show a greater variation. Following the ingestion of 50 gm. of fructose, no change in quotient occurred which could be considered significant in four of the five experiments. The single high quotient of 1.04 for the first period after ingestion of fructose in the first experiment (March 13th) can be discarded on the basis of a high respiratory rate and probable hyperventilation. Therefore the heat production for this period has not been calculated. Thus it appears that little if any stimulus to carbohydrate oxidation occurred in this subject from the ingestion of 50 gm. of fructose. Since Wierzuchowski ('26) obtained a rise in respiratory quotient from phlorhizinized animals after a second dose of glucose which was not demonstrable after the first dose, and similar results were found in normal animals after fasting (Dann and Chambers, '30), a second 50 gm. portion of fructose was taken by subject A. O. 2 to 3 hours after the first dose as noted in table 1. This led to a distinct rise in the quotient to 0.93 on April 3rd and 0.95 on April 10th.

The last experiment (June 26th) disagreed with the other four in that the quotient was maintained at a constant high level of about 0.91 throughout the four periods after the first dose of fructose and for the first two periods after the second dose of sugar. It then returned to the basal level. The technique of this experiment was different from the two preceding ones in that during the experiment blood samples were drawn at the intervals shown in table 3.

Despite the high level of carbohydrate metabolism in the basal periods of this fructosuric subject, 50 gm. of fructose were insufficient to stimulate a significant increase in sugar oxidation, although a second 50-gm. dose was effective.

The basal metabolism was 37.8 calories per square meter per hour or a B. M. R. of —4. The maximum extra heat production following the ingestion of fructose averaged about 4.5 calories per hour in the absence of a definite rise in respiratory quotient, a reaction similar to that found in the impaired

TABLE 3

Urinary excretion of nitrogen and fructose after ingestion of fructose

SUB-JECT	DATE	BASAL N/HR.	FIRST 50 GM. FRUCTOSE				SECOND 50 GM. FRUCTOSE			
			Period	N/hr.	Total fructose	Per cent of ingested fructose	Period	N/hr.	Total fructose	Per cent of ingested fructose
		mg.	hours	mg.	gm.		hours	mg.	gm.	
A.R.	7/17/37	486	[1]	414	[1]	[1]				
A.R.	7/24/37	546	5	384	8.1	16.1				
A.R.	7/31/37	624	5	483	8.2	16.3				
A.R.	8/ 7/37	[2]	4	[2]	7.4	14.8				
A.R.	1/ 8/38	[2]	6	[2]	8.8	17.6				
A.O.	3/13/37	298	3.25	246	5.1	10.2				
A.O.	3/20/37	528	3.50	348	6.9	13.8				
A.O.	4/ 3/37	541	3.5	305	6.8	13.6	2.67	394	7.3	14.6
A.O.	4/10/37	731	2.67	329	5.2	10.4	2.5	300	7.7	15.4
A.O.	6/26/37	403	3.5	408	6.6	13.2	2.67	437	8.3	16.6

[1] Glucose given.

[2] Nitrogen excretion not determined.

glucose metabolism of the fasted normal individual (Chambers, '38).

The lactic acid concentration in the venous blood following the ingestion of fructose by these two subjects (table 2) showed changes of little significance, thus confirming the earlier observations of Blatherwick and of Edhem, Erden and Steinitz. The apparent rise at 3 hours in the second experiment with A. O. (June 26, 1937) was not increased by the administration of more fructose.

The data on blood sugar changes also are shown in table 2. The rise in blood fructose after ingestion was in the range of 40 to 50 mg. per cent and accounts for the observed rise in total sugar, since the blood glucose exhibited little change' or a slight fall. These results are in accord with those of the earlier investigators.

The urinary excretion of fructose by the two subjects as shown in table 3 averaged between 1.5 and 2.0 gm. per hour or a total excretion in 3 to 5 hours of from 10 to 20% of that ingested. The figures for nitrogen excretion, used in the calculation of the respiratory metabolism, are included in table 3.

There was no excretion of glucose by subject A. R. following its ingestion in the experiment carried out on July 17th. Fructose in the basal urines was negligible.

DISCUSSION

The constancy of fructose excretion in the urine by the fructosuric subject indicates that approximately 80 to 90% of ingested fructose may follow the major pathways of normal carbohydrate metabolism, namely, oxidation and glycogen formation. The similarity in the rise in respiratory quotient after fructose and after glucose in subject A. R. (fig. 1) suggests that much of the fructose ingested by this subject may be metabolized as glucose. In the other subject a stimulating dose of fructose was usually needed before the rise in R.Q. appeared.
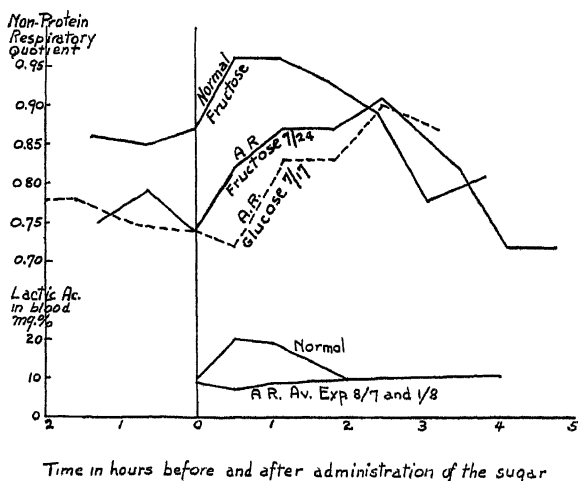
Fig. 1 The effect of ingested fructose and glucose on the respiratory metabolism and blood lactate concentration of normal and fructosuric subjects.

In the metabolism of fructose the normal individual experiences a rapid, marked rise in respiratory quotient approaching or surpassing unity and a marked increase in blood lactate, as illustrated in figure 1 by data from one of the normal control subjects. Blood sugar is only slightly elevated and none of the fructose is excreted in the urine. The early,

marked rise in both the R.Q. and in blood lactate was not found in the fructosuric subjects. A relationship between the elevated blood lactate and the high quotient is suggested by their presence in normal individuals and their absence in the fructosuric subjects.

The rapid rise in respiratory quotient following fructose ingestion by the normal individual has been ascribed either to fat formation (Cathcart and Markowitz, '27), to lactate displacement of blood CO_2 (Campbell and Maltby, '28) or to more rapid carbohydrate oxidation (Carpenter and Lee, '33; Deuel, '36; Carpenter, Bensley, Dill and Edwards, '37). Bachmann and Haldi ('37) found that the correction was a small factor in the total rise in R.Q. when the observed quotients were recalculated for excess CO_2 on the basis of displacement by lactic acid in the blood only (7% of the body weight). In the tissues of rats a rise in lactic acid after fructose ingestion has been noted by Blatherwick et al. ('40). Evidence of a rapid diffusion of lactic acid through the tissues indicates that in calculating the displacement of CO_2 the content of the intracellular tissue fluid in addition to that of the blood should be used, that is, a distribution of lactate through approximately 50% of the body weight.

Applying this correction to the average normal control experiment shown in figure 1, the obtained quotient of 0.97 becomes 0.87 on the basis of a rise in blood lactate amounting to 10 mg./100 cc. during the first $\frac{1}{2}$ hour after fructose. By a similar calculation of the experiment on fructosuric subject A.R. in figure 1, if there had been a blood lactate increase of 100% after fructose ingestion such as occurred in the normal, the first quotient would have been 0.96 instead of the observed value of 0.82. These recalculated quotients from the fructosuric and normal subjects suggest that the rapid rise in respiratory quotient during the first $\frac{1}{2}$ hour after fructose ingestion by the normal subject is related to the increase in lactic acid.

Since the blood lactate concentration returned to the initial level within 2 hours, a compensatory retention of CO_2 should

have occurred in this time. The rising quotient of increased carbohydrate oxidation evidently masked the compensatory fall. Thus over a period of 2 hours the acid-base changes should reach a balance and the respiratory quotient for the whole period should present a true metabolic quotient, comparable to that of the fructosuric subject who exhibits no changes in lactate.

The extent of the impaired oxidation of carbohydrate in fructosuric subject A. R. is seen in a consideration of the 2-hour period after fructose administration. During this time A. R. had an average non-protein respiratory quotient of 0.85 (table 1, 7/24). From the oxygen consumption it is calculated that he oxidized 6.32 gm. of carbohydrate per hour. For the same period the normal subject (fig. 1) had an average quotient of 0.95. On the basis of a normal quotient of 0.95, A. R. would have oxidized 10.05 gm. of carbohydrate per hour, a difference of 3.73 gm. per hour. This difference is adequate to account for the fructose excreted (table 3). The defect in carbohydrate oxidation in subject A. O. (table 1) was more striking, since a stimulating dose of fructose was required in most of the experiments before any increase in carbohydrate oxidation could be demonstrated. Although some investigators have suggested conversion of fructose to fat, the rise in quotient in the data presented here is calculated as carbohydrate oxidation, since no direct evidence for fat formation under these conditions is available.

These data emphasize two abnormal reactions to ingested fructose in the fructosuric subject, namely, a smaller than normal increase in carbohydrate oxidation and the absence of a significant rise in blood lactate. It is probably the former defect which leads to the accumulation of fructose in the blood and the consequent fructosuria, since for the normal lactate changes calculated above only about 2.5 gm. of sugar are needed. No direct relationship between the two reactions is evident. Lactic acid might be an intermediary in the transformation of fructose to glucose or a step in the direct oxidation pathway.

The authors are indebted to Mary E. Ewing and Susan D. Sawyer for the determinations of lactic acid, total sugar and fructose.

SUMMARY

The effect of ingested fructose on the respiratory metabolism, the concentration of sugar and lactic acid in the blood, and fructose excretion have been determined in two fructosuric subjects.

The rapid initial rise in respiratory quotient, characteristic in the normal individual after fructose ingestion, was absent in both subjects. One subject responded with a slow rise in R.Q. similar to that after glucose. The other usually exhibited an increase in quotient only after a stimulating dose of fructose had been taken.

Blood and urine studies also were made of two other subjects. All failed to show the normal rise in blood lactate and excreted between 10 and 20% of the ingested fructose, in agreement with the reports of earlier investigators.

The two defects in fructose metabolism, diminished oxidation and absence of a significant rise in blood lactate, are discussed. It is suggested that the former may account for the observed fructosuria.

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THE CALCIUM REQUIREMENT OF MAN: BALANCE STUDIES ON SEVEN ADULTS [1]

JULIA OUTHOUSE, HERTA BREITER, ESTHER RUTHERFORD, JULIA
DWIGHT, ROSALIND MILLS AND WILLIAMINA ARMSTRONG

*Department of Home Economics, College of Agriculture, University of Illinois,
Urbana*

In the interests of good nutrition, the adult must ingest
a certain amount of calcium every day, even though the forma-
tion of new bone tissue has long since ceased—even though
his skeletal tissues may already be well calcified. This need
is the result of a constant daily drain of calcium from the
body by way of the kidneys and intestines; if insufficient
quantities of calcium are ingested habitually, this daily loss
will eventually result in a depleted skeleton. Just how much
calcium is necessary to prevent the occurrence of such a
deficit has been critically evaluated by Sherman ('20), Leitch
('37) and Mitchell and Curzon ('39). Although these investi-
gators made use of the same type of material, i.e., previously
reported calcium metabolism data on adults, they employed
different methods for their evaluations; hence, it is not sur-
prising that they reached widely differing conclusions con-
cerning the adult's minimal requirement for calcium. Mitchell
and Curzon, in contrast to the two other investigators, pointed
out a relation between the need for calcium and the size of the
body and concluded that, as an average, an intake of 9.75 mg.
per kilogram body weight by the adult would be sufficient to
insure calcium equilibrium. Moreover, they believed that
such a requirement was consistent with an average rate of
utilization of dietary calcium of 30%. If Mitchell and Curzon's

figure for utilization is physiologic for man, then Sherman's failure to realize this may be the explanation for his low figure for requirement; i.e., 450 mg. for a 70 kg. person. However, until 1939, when Kinsman et al. and Steggerda and Mitchell published their studies on calcium utilization, there was no experimental proof of man's limited ability to make use of calcium. Furthermore, at the time the present study was undertaken only one adult, the man studied by Steggerda and Mitchell, had actually been subjected to an experiment which was specifically designed to reveal both the utilization of and the requirement for calcium; this man utilized 20% of the calcium of milk and had a total requirement of 9.2 mg. per kilogram. Obviously, more experimental data of this nature are necessary before a universally acceptable figure for calcium requirement can be obtained; for this reason the study reported here was undertaken.

EXPERIMENTAL

This study makes use of data presented in the earlier paper dealing with the calcium balances of seven healthy adults and their ability to utilize milk calcium (Breiter, Mills, Dwight, McKey, Armstrong and Outhouse, '41), and reference should be made to that work for detailed information concerning the experiment. The subjects were four women and three men—ranging in age from 21 to 42 years; they were allowed to carry on their usual activities and, at all times, reacted satisfactorily, psychologically as well as physiologically, to the conditions of the experiment. The basal dietary, which was presumably adequate in all nutrients except calcium, contributed approximately 270 mg. calcium daily. To this dietary, milk was added in quantities almost sufficient to induce calcium equilibrium, the daily amounts judged to be necessary being 180 gm. for subject Jd, 220 gm. for Jo, 260 gm. for Hb, Ws and Mh, 260 gm. (as an average) for Bm and 500 gm. for Rd. Thus, the proportion of milk calcium to non-milk calcium was practically uniform for six of the seven subjects; actually the ratios were 1.6, 1.7, 1.6, 1.4, 1.6, 1.3 and 2.7 to 1 for

subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively. The milk-supplemented dietary was fed for 19 to 34 consecutive days.

RESULTS

The data which are pertinent to the assessment of the calcium requirement of these seven subjects are given in table 1; they include the quantity of calcium eaten, the resulting calcium balances and the rate at which each subject utilized the calcium of milk. On the respective daily calcium intakes of 574, 568, 498, 603, 580, 451 and 873 mg., subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd had calcium balances of +24, +5, —39,

TABLE 1

The calcium requirement of seven adults

Subjects	Hb	Bm	Jo	Ws	Mh	Jd	Rd	Ave.
Weight in kg.	61	55	64	65	60	53	74	
Height in cm.	161	167	161	171	166	166	181	
Surface area in sq. m.[1]	1.64	1.61	1.66	1.75	1.64	1.57	1.93	
Total calcium intake (ave. daily) in mg.	574	568	498	603	580	451	873	
Calcium balance (ave. daily) in mg.	+24	+5	—39	—5	—13	—36	—22	
Utilization of milk calcium, %	35.1	30.6	30.3	20.1	17.6	15.3	20.4	24.2
Calcium requirement (total daily) in mg.	506	552	627	628	654	686	981	662
Calcium requirement (per kg.) in mg.	8.3	10.0	9.8	9.7	10.9	12.9	13.3	10.7
Calcium requirement (per cm.) in mg.	3.1	3.3	3.9	3.7	4.0	4.1	5.4	3.9
Calcium requirement (per sq.m.) in mg.	309	344	376	359	400	438	508	391
Milk requirement [2] (total daily) in gm.	236	281	360	313	353	405	615	

[1] Surface area was determined by the method of Boothby and Sandiford ('21).

[2] These are the quantities of milk which the subjects, in order to be in calcium equilibrium, would have to add to the non-milk foods used in this experiment.

—5, —13, —36 and —22 mg. (The wide deviation from zero shown in the balances indicates the impossibility of predicting the exact calcium requirement of a given individual.) The utilization of the calcium of milk by these seven subjects amounted to 35.1, 30.6, 30.3, 20.1, 17.6, 15.3 and 20.4%, respectively. These latter figures have been used in calculating the extent to which each subject's calcium intake would have had to be increased or decreased in order to bring about exact calcium equilibrium. Such a procedure seems reasonable particularly since none of the calcium balances departed radically from zero.

The daily calcium requirements of the subjects were computed by applying to the above data the following formula,

$$\text{Calcium Requirement} = \text{Calcium Intake} \pm \left(\frac{\text{Calcium balance}}{\% \text{ Utilization of milk calcium}} \times 100 \right),$$

the plus sign being used for the subjects who were in negative balance, the minus sign for those in positive balance. The resulting values of 506, 552, 627, 628, 654, 686 and 981 mg. were obtained for subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively. The average requirement for the group was 662 mg. of calcium.

<center>DISCUSSION</center>

The values reported here refer to the minimal quantity of dietary calcium needed by these seven adults for the maintenance of calcium equilibrium. Their range is great, i.e., from 506 to 980 mg. In an attempt to determine the cause of this variability, the data were evaluated in a number of ways. When converted to the conventional weight basis they became 8.3, 10.0, 9.8, 9.7, 10.9, 12.9 and 13.3 mg. per kilogram for subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively, and averaged 10.7 mg. Obviously, if weight were the only determinant of calcium requirement, one would expect to find strictly similar values for all subjects instead of the wide range given here. It may be significant that both Jd and Rd, who represented the extremes in weight (i.e., 53 and 74 kg., respectively), had the same per-kilogram requirement

of approximately 13 mg.; on the other hand, subjects Bm, Jo and Ws also had virtually identical, but considerably lower, requirements (10.0, 9.8 and 9.7 mg. per kilogram, respectively). Less variability might have been found if all subjects had been equally well-padded with fat; thus the high requirement of Jd and Rd might be explained on the premise that their skeletal tissues represented a larger proportion of their weight than was true for subjects Hb, Jo and Ws who had a lower per-kilogram requirement, were it not for the fact that subjects Bm and Mh were "lean" individuals. In an attempt to rule out this factor, the total requirements were expressed on the basis of recumbent length, with resulting values of 3.1, 3.3, 3.9, 3.7, 4.0, 4.1 and 5.4 mg. per centimeter for the subjects in the usual order. On this basis, Jd's requirement of 4.1 mg. becomes more like those of Mh, Jo and Ws, who needed 4.0, 3.9 and 3.7 mg., respectively, whereas Rd again shows an extremely high requirement. This approach, like the former, yields considerable variability. The same statement can be made when the data are expressed on the basis of surface area —the values ranging from 309 to 508 mg. per square meter. To what extent the variability in requirement may have been determined by the subjects' ability to utilize calcium is not entirely clear. Although Bm and Hb, two of the best calcium utilizers, had the lowest requirements, Jo, who utilized calcium equally as well as these two subjects, had a requirement similar to those of Ws, Mh and Jd—none of whom could make use of more than 20% of his dietary calcium. Obviously, the ability of these persons to utilize calcium was not the sole determinant of their requirement—neither was their height, their weight, nor their surface area.

The average requirement for calcium (i.e., 662 mg.) computed for these seven adults is higher than those heretofore published. The 10.7 mg. per kilogram average more nearly approaches the 9.75 mg. value obtained by Mitchell and Curzon ('39) than it does the figures of Sherman ('20) or of Leitch ('37); it differs from Mitchell and Curzon's primarily in that their calculations would assign to subjects Jd and Rd, re-

spectively, 151 and 258 mg. less calcium than they actually required. Although the standard of 550 mg. recommended by Leitch (for either men or women, regardless of size) would meet the needs of subjects Bm and Hb, its application would result in serious calcium shortage for the other five—a shortage amounting to 430 mg. daily for subject Rd. On comparing these figures with that of Sherman, it is apparent that every one of the subjects had a requirement in excess of 450 mg. and that the average for the group is almost 50% greater. Sherman's figure is predicated on the basis of a body weight of 70 kg., and, when the requirements of the seven subjects are calculated according to this weight, the average becomes 752 mg., which is 67% greater than Sherman's 450 mg. figure and 10% greater than his recommended dietary standard of 680 mg.—obtained by increasing the 450 mg. value by 50% "as a margin of safety."

The reason for this disparity in calcium requirement, as determined by these two methods, warrants elucidation—especially since the same type of data (i.e., calcium balances) is used in both studies. Sherman's method (Sherman, Mettler and Sinclair, '10) was based on the sound reasoning that, inasmuch as an adult needs to eat only enough calcium to equal that which is excreted by the kidneys and intestines, his requirement can be determined by determining his total calcium excretion and that ". . . . only those experiments in which there was a reasonably close approach to equilibrium of lime . . . . can be taken as indicating the lime . . . . requirement." However, very few of the many adults studied under experimental conditions have been virtually in calcium equilibrium; Sherman himself made use of balance data which deviated radically from zero. In believing that the total calcium excretion in such cases is synonymous with requirement, he really was implying that adults could utilize 100% of their dietary calcium; e.g., if an individual is in negative balance to the extent of 150 mg., he would need to add only 150 mg. to his intake in order to be in calcium equilibrium. In

1920, this assumption was not unreasonable; today it must be appreciated that such utilization is probably never realized by man—from 15 to 35% was the range for the seven subjects of this study, and 30% was the average rate of utilization found by Mitchell and Curzon. Such an assumption does not complicate the present study—nor does it enter into Mitchell and Curzon's evaluation of the adult's requirement for calcium.

The extent to which this factor of calcium utilization will modify figures for requirement can be seen by reevaluating the data presented in the two studies. By the Sherman method, the requirement for subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively, would be 550, 562, 537, 609, 593, 487 and 895 mg. and would average 605 mg. These values do not differ greatly from those computed on the basis of each one's individual capacity to utilize calcium—the reason being that the calcium balances of these subjects were not far from zero. But many of Sherman's subjects were losing more than 100 mg. of calcium daily, and, hence, their actual requirements must have been in excess of those which he assigned to them. If one assumes—in the absence of specific data—for each of his subjects a utilization rate of 30% (i.e., Mitchell and Curzon's figure derived from data on 107 adults, including these eighteen), one obtains the results recorded in table 2. In preparing this table, the balances for all periods for a given subject were averaged—hence the eighteen sets of data, rather than Sherman's original ninety-seven. For those subjects (nos. 3 through 75) who reacted to the low calcium intakes in a manner similar to that exhibited by the subjects of the present study, the average calcium requirement, computed at a 30% utilization rate, was 769 mg. in contrast to the average of 494 mg. obtained by Sherman's method. For those who were virtually in equilibrium or storing calcium (1, 2 and 76 through 95) the Sherman values are the higher, but these subjects must have utilized more than 30% of their dietary calcium in order to have been in equilibrium at such

TABLE 2

Calcium requirement of Sherman's ('20) subjects as computed by two methods

DESIGNATION OF SUBJECT		WEIGHT	CALCIUM INTAKE	CALCIUM BALANCE	CALCIUM REQUIREMENT		UTILIZATION RATE USED IN COMPUTATION [1]
In table 1 Sherman ('20)	By original investigator				Computed on basis of 70 kg.		
					By Sherman's method	By method used in this study	
		kg.	mg.	mg.	mg.	mg.	%
1, 2	Bertram (1878)	70	275	—6	285	295	30 [2]
3	Renvall ('04)	71	860	+28	820	756	22
4, 5	von Wendt ('05), subject G	72	195	—207	385	592	50
6	Holsti ('10)	58	359	—220	660	1026	33
7, 8, 9	Sherman, Mettler and Sinclair ('10)	62	465	—156	577	985	30
10–15, inc.	Sherman ('20), subject I	61	390	—140	613	980	30
16–24, inc.	Sherman, Gillett and Pope ('18), subject L	57	280	—46	409	532	30
25–34, inc. } 49–55, inc. }	Sherman, Gillett and Pope ('18), subject K	52	288	—88	528	782	30
35–41, inc.	Sherman, Wheeler and Yates ('18), subject M	59	140	—110	307	602	30
42–48, inc.	Sherman, Wheeler and Yates ('18), subject N	55	250	—100	450	742	30
56, 57, 58	Sherman ('20), subject E	69	323	—130	330	495	76
59–63, inc.	Sherman ('20), subject R	80	212	—114	285	518	30
64–68, inc.	Sherman and Winters ('18), subject O	54	330	—110	567	1075	23
69–75, inc.	Sherman, Winters and Phillips ('19), subject P	67	303	—171	497	910	30
76–81, inc.	Rose ('20), subject E.D.B.	54	349	+42	400	280	30 [2]
82–89, inc.	Rose ('20), subject R.S.E.	56	291	+17	416	234	30 [2]
90, 91, 92	Rose ('20), subject E.H.	45	261	+10	383	350	30 [2]
93, 94, 95 [3]	Rose ('20), subject E.W.	48	300	+82	323	28	30 [2]

[1] Calcium utilization rates could be computed for only five subjects; for the others a rate of 30% was assumed.

[2] Obviously, the rate of calcium utilization must have been greatly in excess of 30%.

[3] The data for nos. 96 and 97 could not be located in the literature.

low levels of intake.[2] Moreover, the fact that Rose's subjects (i.e., nos. 76 to 95) were able to store calcium suggests that they may have been in a condition of poor calcium nutrition prior to the beginning of the experiment; such a condition might have resulted in more efficient utilization of calcium— if Rottensten's ('38) findings on rats can be applied to man. If these individuals had utilized more than 30% of their calcium, then their true requirements would lie somewhere between the values computed by these two methods.

In the present study, the requirement for calcium has been determined from data collected while the seven subjects were ingesting a fixed dietary. The computed values, therefore, can apply to these individuals only while they are on dietaries which are similar to the one used in this study with respect to the distribution and quantity of non-milk foods and identical with it in the proportion of milk calcium to non-milk calcium. The latter consideration is important, inasmuch as there is some evidence [3] that the calcium of plant foods may not be as well utilized as is the calcium of milk. Obviously, then, if these individuals were to obtain a significantly larger proportion of their dietary calcium from non-milk sources, the quantities of calcium designated here as the minimal requirement might be insufficient. Under the conditions of this experiment the amount of milk calcium needed for equilibrium by subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively, would represent 57, 62, 70, 60, 65, 71 and 76% of their total requirement. Translated into terms of fluid milk, these percentages indicate daily requirements of 236, 281, 360, 313, 353, 405 and 615 gm. in addition to the non-milk foods of the

[2] Such extraordinary balances coincident with a meager intake of calcium were never encountered during the present study; if they are the result of an adaptation to a low calcium dietary (Nicholls and Nimalasuriya, '39), the process of adjustment must have taken a relatively long time, inasmuch as Mh and Ws, who had subsisted on a calcium-poor dietary for at least 3 years prior to the beginning of the experiment (Breiter et al., '41), were unable to attain calcium equilibrium at comparably low levels of intake.

[3] Unpublished data reveal that although two of these subjects utilized as much of the calcium of carrots as they did that of milk, the other five utilized far less.

basal dietary. Practically speaking, then, six of these adults could have attained calcium equilibrium on less than 1 pint of milk daily, but, on such a milk allowance, subject Rd would have drained calcium from his skeleton at the rate of 32 mg. each day.

SUMMARY ANND CONCLUSIONS

This study reports the calcium requirement of seven adults ranging in age from 21 to 42 years. The requirement was computed from data on calcium balances secured at levels of intake almost sufficient to induce calcium equilibrium and from data concerning the extent to which each subject could utilize milk calcium. Balances of +24, +5, —39, —5, —13, —36 and —22 resulted when the respective subjects ingested 574, 568, 498, 603, 580, 451 and 873 mg. of calcium daily. Since these subjects could utilize, respectively, only 35, 31, 30, 20, 18, 15 and 20% of the calcium of milk, the following adjustments in calcium intake would have been necessary in order to bring about calcium equilibrium: —68, —16, +129, +25, +74, +235 and +108 mg. The total respective requirements, therefore, would be 506, 552, 627, 628, 654, 686 and 981 mg. calcium daily; they average 662 mg. which, when it is based on weight, height and surface area, amounts to 10.7 mg. per kilogram, 3.9 per centimeter and 391 per square meter.

These requirements, calculated on a 70-kg. weight basis, average 752 mg.—which is 67% greater than Sherman's 450-mg. requirement. But the latter figure is too low because it is predicated on the assumption that adults utilize 100% of their dietary calcium.

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FURTHER EXPERIMENTS ON THE CALCIUM REQUIREMENT OF ADULT MAN AND THE UTILIZATION OF THE CALCIUM IN MILK [1]

F. R. STEGGERDA AND H. H. MITCHELL

Department of Physiology, and Division of Animal Nutrition, University of Illinois, Urbana

The experiments reported in this paper are a continuation of those previously reported (Steggerda and Mitchell, '39) on the same subject. The same methods of study have been used and need not be repeated in detail here.

The general plan was to place each subject upon a basal diet containing on the average 203 mg. of calcium daily. The general nature of the basal diet was the same as that already described except for the use of a somewhat wider range of foods, mainly low-calcium foods, to satisfy the larger number of subjects involved. Besides the foods contained in the previous diet, the following were employed in varying amounts, either as substitutes for other foods or as additions to the diet: farina, rice, egg, liver, corn, carrots, tomato juice, apricots, peaches, bananas and lettuce. Supplements of vitamins A and D were taken daily. After 4 or 5 days on a constant intake of food, collections of feces and urine were made in successive 4-day periods until it was apparent that the subject was adjusted to this low level of calcium nutrition. Carmine was used as a feces marker.

In succeeding metabolism periods, supplements of milk products were added to the basal diet in amounts to provide

enough calcium for approximate body equilibrium. The milk products tested included liquid skim milk, dried skim milk ("dry milk solids"), liquid whole milk and homogenized milk. During these periods, small adjustments were made in the basal diets to equalize the energy intake in basal and test periods. All experimental periods consisted of a succession of 4-day collection periods (very occasionally 5-day periods) continued until it was evident that an adjustment to the experimental diet had occurred.

There were nine male adult subjects, consisting of graduate students and staff members, varying in age from 24 to 39 years. The ages, body weights, body heights and surface areas of the subjects are given in table 1. Throughout the experiment they remained in good health and maintained their body weights.

TABLE 1

Description of experiment subjects

SUBJECT	AGE	BODY WEIGHT	HEIGHT	BODY SURFACE [1]
	yrs.	*kg.*	*cm.*	*m²*
Ha	24	69.8	186	1.93
Sp	30	72.6	175	1.82
Na	39	63.5	169	1.73
Fa	29	55.8	169	1.64
Cl	38	62.6	175	1.76
St	36	82.5	180	2.02
Fo	24	65.8	171	1.77
Ma	26	79.4	182	2.01
Ne	27	74.8	186	1.99

[1] Estimated by the DuBois height-weight formula (DuBois and DuBois, '16).

EXPERIMENTAL RESULTS

The experimental results are summarized in table 2. It seems unnecessary to report the calcium metabolism data for the individual 4-day periods. Suffice it to say that in the twenty-five experimental periods, the average standard deviation of the 4-day calcium balances about the mean balance for the period was 53 mg.

TABLE 2

The calcium metabolism data and estimates of utilization and requirement based on them

SUBJECT AND BODY WEIGHT IN KG.	CALCIUM SUPPLEMENT TESTED	TEST PERIOD	AVERAGE DAILY METABOLISM OF CALCIUM					UTILIZATION OF SUPPL. CALCIUM	INDICATED Ca REQUIREMENT:	
			Ca intake		Ca excreted		Balance		Per kg. body weight	Per m² body surface
			Basal diet	Suppl. food	In feces	In urine				
		days	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*%*	*mg.*	*mg.*
Ha 69.8	None	20	187	0	212	173	−198	...	...	...
	Dry milk solids	24	198	543	513	193	+35	42	9.3	341
	Liquid skim milk	20	186	337	353	190	−20	53	8.0	291
	Dry milk solids	20	209	337	390	166	−10	52	8.2	293
Sp 72.6	None	20	176	0	173	180	−177	...	...	...
	Dry milk solids	16	188	337	309	266	−50	36	9.4	376
Na 63.5	None	16	187	...	178	75	−66	...	...	...
	Dry milk solids	20	166	337	392	110	+1	21	7.8	288
	Liquid skim milk	20	199	337	434	104	−2	18	8.6	316
Fa 55.8	None	32	165	0	168	76	−79	...	...	...
	Dry milk solids	32	172	410	459	113	+10	21	9.5	324
Cl 62.6	None	28	215	0	191	146	−122	...	...	...
	Dry milk solids	20	272	528	616	198	−14	18	14.0	499
St 82.5	None	20	195	0	216	82	−103	...	...	...
	Liquid whole milk	16	213	431	548	105	−9	21	8.3	340
	Homogenized milk	20	213	438	558	106	−13	20	8.7	354
Fo 65.8	None	12	224	0	295	83	−154	...	...	...
	Homogenized milk	16	211	435	595	85	−34	28	11.7	433
Ma 79.4	None	20	258	0	263	98	−103	...	...	...
	Liquid whole milk	20	227	430	487	142	+28	33	7.2	285
	Homogenized milk	20	290	438	556	128	+44	31	7.4	292
Ne 74.8	None	16	222	0	278	52	−108	...	...	...
	Liquid whole milk	21	254	430	589	67	+28	29	7.9	295
	Homogenized milk	21	245	438	609	64	+10	26	8.6	322
	Homogenized milk	20	252	420	619	69	−16	20	9.8	378
Averages								29	9.55 [1]	357 [1]

[1] These values are the averages of the averages for the nine experimental subjects.

In the test periods of the experiment, the supplemental calcium from the milk products contributed an average of 65.5% of the total dietary calcium. The excretion of calcium occurred mainly in the feces: In the basal periods an average of only 33% of the total output of calcium appeared in the urine, while in the test periods, only 24% so appeared.

The utilization of the supplementary calcium was computed by determining its sparing effect on the excretion of body (endogenous) calcium. For example, subject Ha in his basal low-calcium period lost daily from his body 198 mg. of calcium. In the following period his calcium intake was increased by 554 mg., a supplement of "dry milk solids" accounting for 543 mg. As a result of this added intake, the loss of calcium from the body was entirely obviated and a slight storage of calcium, 35 mg. daily, brought about. Thus, the increased intake of calcium, 554 mg., spared 198 mg. of body calcium and induced a storage of 35 mg. The utilization of the supplemental calcium may be computed according to the following formula:

$$[(198 + 35) \div 554] \times 100 = 42.$$

Thus, the percentages in column 9 of table 2 measure the sparing effects of the supplemental forms of calcium with reference to the endogenous losses of calcium from the body, taking due account also of any storages of calcium, which were purposely either prevented entirely or kept at minimal levels. While considerable variation exists among these percentages of utilization, the variation seems to be the result mainly of differences in individual performance. Subject Ha was consistently high in his utilization of milk calcium, his average utilization being 49. Subject Ma was intermediate with utilizations of 31 and 33%, while subjects Na and St were consistently low. It is interesting to compare the performance of the latter subject in this experiment with his performance in the previous experiment (Steggerda and Mitchell, '39), in which he was the sole subject. In the present experiment he utilized the calcium of liquid whole milk to the extent of 21% and the calcium of homogenized milk to the extent of 20%.

In the former experiment he utilized the calcium in "dry milk solids" and in calcium gluconate to the same extent, i.e., 20%.

The results of this experiment do not afford any basis for assuming differences in the biological value of calcium among the different milk products tested. The data for subjects Ha and Na reveal no significant difference between liquid skim milk and "dry milk solids" in this respect, while the data for subjects St, Ma and Ne indicate no difference between liquid whole milk and homogenized milk. While the average utilizations for the three products, 32 for "dry milk solids," 31 for liquid milk (both whole and skim) and 25 for homogenized milk, might indicate an inferiority in the biological value of calcium in homogenized milk, the lower average for this product is largely a result of the fact that subject Ha, the highest utilizer of calcium in the group, was not used in testing homogenized milk. The average utilization of 29% seems applicable to all the milk products tested.

The calcium requirements of the subjects were estimated by computing the amount of dietary calcium required for equilibrium. Such estimations could be made only in those periods during which supplemental calcium was fed. An illustration of the method can be taken from the data of the second experimental period for subject St. In this period, an intake of 644 mg. of calcium induced an average daily deficit of 9 mg. Since the supplemental calcium was being utilized to the extent of 21%, the deficit of 9 mg. could presumably be erased by increasing the intake by 43 mg. $(9 \div .21 = 43)$, making the estimated requirement 687 mg. Slight positive balances of calcium have been corrected for by deducting from the intake an amount of supplemental calcium estimated by the same method, to be the dietary equivalent of the observed balance. All estimates of calcium requirements have been expressed both in milligrams per kilogram of body weight (column 10), and in milligrams per square meter of body surface (column 11). Obviously these requirements relate to the particular sources of calcium included in the experimental diets, which were all characterized by containing milk products

in amounts to furnish about two-thirds of the content of calcium. To the extent that other sources of calcium would be differently utilized, to that extent the above calculated requirements of calcium for adult maintenance would be modified. However, since milk calcium appears to be as efficiently utilized as any form of dietary calcium, any modification of the requirements calculated above, necessitated when other diets are used, would in all probability raise the estimates. Hence, these estimates may be regarded as minimal in all probability.

The average calcium requirements are 9.55 mg. per kilogram of body weight, or 357 mg. per m^2 of body surface. The requirements are expressed in these two ways in recognition of the fact that no one method of expression has been established as superior to all others. Evidence for believing that the calcium requirement of maintenance varies with the body weight raised to a power less than 1, if not with the surface area, has been developed elsewhere (Mitchell and Curzon, '39). Expressed to the m^2 of body surface, the calcium requirements in table 2 are somewhat less variable than when expressed to the kilogram of body weight, the coefficients of variation being 19.1 and 21.6, respectively. However, the difference in variability is too small to constitute a weighty argument in favor of the former method of expression. The problem involved here is sufficiently important to merit further, and more direct, study.

<center>DISCUSSION</center>

The finding in these experiments that the commercial desiccation of milk does not appreciably affect the biological value of its calcium is in agreement with the results obtained in rats in a publication from this laboratory (Shields et al., '40).

The calcium requirement for adult maintenance indicated by this study, i.e., 9.55 ± 0.46 mg. per kilogram of body weight and 357 ± 15 mg. per m^2 of body surface, is considerably higher than the estimate of Sherman ('20), i.e., 0.45 gm. per 70 kg. body weight, or 6.4 mg. per kilogram. It is in good agreement, however, with the estimate of Leitch ('36-'37),

obtained from a much larger selection of published experiments, i.e., 0.55 gm. per woman subject with an average body weight of 55.6 kg., equivalent almost exactly to 10.0 mg. per kilogram of body weight. It is also in good agreement with the value of 9.75 mg. per kilogram body weight, deduced more recently by Mitchell and Curzon ('39) from a still larger selection of experiments on the calcium metabolism of adult human subjects.

In view of the importance attached to Sherman's estimate of the calcium requirement of adult man, and of the preponderate position it occupies in past and current nutritional writings, it seems necessary in evaluating the much higher figure obtained in this experiment to inquire into the cause for the great discrepancy between the two.

Sherman's estimate was derived by averaging the calcium output, reduced to a common body weight of 70 kg., observed in ninety-seven balance periods of 3 to 8 days' duration upon a much smaller, but unstated, number of adult subjects. The following quotation from Sherman's article is pertinent in this connection:

Experiments made to test calcium requirements have sometimes involved the use of diets furnishing so little calcium that the output (although greater than the intake) has been depressed below the point at which equilibrium could actually be maintained. Largely for this reason it is probable that some of the data for indicated calcium requirement in table 1, based as they are on the data of output when the intake was somewhat insufficient, are appreciably below the true requirements of the respective subjects. Doubtless there are also cases in which the requirement was overestimated through the use of diets unnecessarily rich in calcium for the purpose of the test: but these appear to be fewer in number and the general average of 0.45 gm. of calcium (equivalent to 0.63 gm. of CaO) is probably not above the true requirement.

It is to be noted that Sherman's data of calcium output were not corrected to calcium equilibrium, as were the data of this experiment. This would, in fact, have been impossible for most, if not all, of the experiments compiled by Sherman,

since few if any of them were planned to afford information as to the percentage utilization of the calcium in the foods tested. The inability to make such a correction would not lead to serious error in the estimate of requirement if the compilation included about as many experiments in which the calcium balance was positive as experiments in which it was negative, and if the positive balances were as great on the average as the negative ones. But the data were not selected in that fashion. The large majority of the data compiled were taken from metabolism periods in which negative calcium balances were secured, and in those few periods where the balances were positive, they were comparatively small in magnitude. On the contrary, many of the negative balances were large, some even exceeding 200 mg. of calcium daily. Thus, subject R (Sherman, '20) during five consecutive 3-day periods excreted 320, 320, 310, 340, and 340 mg. of calcium daily and his calcium requirement per 70 kg. body weight is entered in the table (nos. 59 to 63 inclusive) as 280, 280, 270, 290 and 300 mg. daily. But during these periods the calcium balances were all negative to the extent of 90, 110, 110, 130 and 130 mg. daily. If the output of calcium is determined in part by the amount of calcium consumed, then to bring this subject into calcium equilibrium would require an increase in his intake of calcium which would in turn induce an increase in the calcium output. Hence, the observed calcium output does not correspond to the requirement of dietary calcium, and must in fact be considerably less than the maintenance requirement.

Sherman's method of computing calcium requirements for maintenance seems to imply that the output of calcium is independent of the intake, at least for wide ranges of intake. This in turn implies that within limits around the point of equilibrium the calcium intake is utilized completely by the human body. But the graphical presentations of the relationship between intake and output of calcium by adult human subjects as given by Leitch ('36–'37) and later by Mitchell and Curzon ('39) indicate clearly a close correlation. In the

latter case, the correlation is measured by a coefficient of +0.800 and a regression equation

$$y = 0.683x + 3.094$$

where y is the output of calcium daily per kilogram of body weight, and x is the intake similarly expressed. In the present experiment also a close relationship is indicated. Thus, in the basal periods where the diet provided only 2.93 mg. of calcium per day per kilogram of body weight, on the average, the output of calcium averaged 4.70 mg. per kilogram. In the other periods on higher levels of calcium, averaging 8.96 mg. per kilogram daily, the output of calcium was also much higher, averaging 8.95 mg. per kilogram. Considering the abnormality of the distribution of intakes in this experiment, the regression of output on intake seems best expressed by the equation

$$y = 0.687x + 2.69$$

x and y having the same significances as above defined.

It thus seems clear that Sherman's calcium requirement of maintenance in man is too low, because of the method by which it was derived, and this error in method probably accounts for the difference between his estimate and that of Leitch, of Mitchell and Curzon, and of this experiment, all of which agree well.

However, it appears that the significance of any average dietary calcium requirement, no matter how impeccable its method of derivation may be, is uncertain when applied to individual cases. Individual variability in this respect on the same type of diet seems to be extreme, as the present experiment clearly shows. Change in the sources of dietary calcium may be expected to change the dietary requirement inversely as the percentage utilization varies. Hence, any indicated requirement applies strictly only to the sources of calcium actually used in the estimation.

The common procedure of estimating the prevalence of calcium undernutrition in a population by comparing the estimated calcium content of individual or family dietaries (an estimation subject to large error) with an average re-

quirement, can yield judgments of extremely dubious validity, not only because of individual variability in requirement and of differences in the utilization of the calcium of different foods, but also because of the great adaptive powers of the human organism. It appears that in the presence of an inadequate supply of any nutrient, including calcium, the body can adjust itself to the situation, either by a more economical use of what little is available, or by a lowering of its own requirements, so that eventually it comes into equilibrium with the limited food supply. Only during this adjustment period can the body be adjudged undernourished, since only in this period is the body suffering a loss of nutriment. When adaptation is complete, the body replaces from its restricted supply all losses of the nutrient from its body, and unless some subsidiary ill effects ensue, it may reasonably be regarded as adequately supplied with food.

Instances of the adaptation of the body to low-calcium regimes are given by Nichols and Nimalasuriya ('39) in Ceylon and by Owen and Irving ('40) among the indigent of Scotland. In the latter article, instances of calcium equilibrium, or near equilibrium, were observed on intakes of 3 to 4 mg. of calcium daily per kilogram of body weight to which the subjects had become accustomed over a period of years. These subjects were beyond middle life and many of them showed evidences of osteoporosis (x-ray examination), which probably developed during the period of adjustment to the low level of calcium intake occasioned by their economic status. An earlier study by Owen ('39) may be interpreted in much the same way, although the calcium restriction was not so severe and the data are not so complete with reference to the description of the subjects. The observations of Basu, Basak and Sircar ('39) on three adult subjects in India consuming vegetarian diets based on rice and whole wheat, very low in their calcium content, indicate for two of the subjects, a successful adaptation to this restricted calcium supply.

Possibly all these cases, even after successful adjustment to the low intake of calcium, are not in the best condition of

health—certainly their dietary is not one that can be recommended—but their calcium balance sheets afford no evidence of malnutrition or no suspicion that malnutrition will eventuate. Nor can their nutritive state be appraised by the mere comparison of their consumption of calcium with an average requirement deduced from balances on experimental subjects that have not experienced a long period of subsistence on diets containing these low quantities of calcium. In such cases objective evidence of malnutrition or ill-health should be forthcoming before an adverse judgment is passed.

<div align="center">SUMMARY AND CONCLUSIONS</div>

Twenty-five calcium balance periods, extending over 12 to 32 days each, were carried out on nine adult men. The first period on each subject involved the testing of diets containing only an average of 203 mg. of calcium daily, but otherwise complete. In subsequent periods the basal diet was supplemented with milk products (liquid whole milk, liquid skim milk, "dried milk solids" and homogenized milk) in amounts to provide enough calcium for approximate equilibrium. Comparisons of the data secured in basal and test periods permitted the estimation, first, of the percentage utilization (biological value) of the supplemental calcium for maintenance, and, second, of the requirement of dietary calcium for equilibrium, i.e., the calcium requirement of maintenance.

No evidence was obtained that the calcium in the different milk products studied was utilized with different degrees of efficiency. An average utilization of 29% was observed, although considerable individual variability existed in this respect, the individual averages being, in increasing order, 18, 19, 21, 21, 25, 28, 32, 36 and 49. In particular, it seems clear that the commercial desiccation of milk, or its homogenization, does not appreciably impair the biological utilization of its calcium.

The average calcium requirement for equilibrium (maintenance) was 9.55 ± 0.46 mg. daily per kilogram of body weight, or 357 ± 15 mg. per m² of body surface. This requirement

relates to diets in which about two-thirds of the calcium content is provided by milk products. It agrees well with the estimate of Leitch ('36–'37) and that of Mitchell and Curzon ('39), deduced from compilations of published data, but it is 50% higher than the estimate of Sherman ('20).

Sherman's estimate of the calcium requirement of maintenance in man is too low by reason of the method of derivation, which neglects to consider the close positive correlation between the intake and the excretion of calcium.

The dangers attending the use of any average calcium requirement of maintenance in assessing the prevalence in a community or population of calcium undernutrition are discussed.

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THE DISTRIBUTION OF PYRIDOXINE (VITAMIN B₆) IN MEAT AND MEAT PRODUCTS [1]

LaVELL M. HENDERSON, HARRY A. WAISMAN AND C. A. ELVEHJEM

Department of Biochemistry, College of Agriculture,
University of Wisconsin, Madison

In previous reports from this laboratory values for the thiamine (Mickelsen, Waisman and Elvehjem, '39 a), riboflavin (Mickelsen, Waisman and Elvehjem, '39 b), pantothenic acid (Waisman, Mickelsen and Elvehjem, '39) and nicotinic acid (Waisman, Mickelsen, McKibbin and Elvehjem, '40) content of meat and meat products have been given. In this paper we wish to report values for the pyridoxine content of animal tissues.

Studies of this kind appear especially advisable in view of recent reports which claim the successful use of this vitamin in treating pellagrins that do not respond completely to the thiamine, riboflavin and nicotinic acid therapy (Spies, Ladisch and Bean, '40; Spies, Bean and Ashe, '39). A definite response has also been observed in the macrocytic anemia of pellagrins and in pernicious anemia patients in relapse (Vilter, Schiro and Spies, '40). Smith and Martin ('40) have reported its use in the treatment of cheilosis. The availability of the crystalline vitamin simplifies the bioassay procedure and allows the values to be expressed in terms of the pure pyridoxine.

The biological assay methods thus far reported (Lunde and Kringstad, '38; Schneider, Ascham, Platz and Steenbock, '39; Wilson and Roy, '38; György, '35; Dimick and Schreffler, '39;

Halliday and Evans, '37) are based on the cure of acrodynia or upon a combination of growth and relief of symptoms in rats. Edgar, El Sadr and Macrae ('38) have described an assay based on growth responses. Complete cure of acrodynia is not effected by pyridoxine alone, but at least one other factor, the "essential fatty acids," is concerned in curing the syndrome (Birch, '38; Schneider, Steenbock and Platz, '40). Furthermore, in most cases the diets used were incomplete since suboptimum growth was obtained even with the administration of the vitamin B_6 concentrate or crystalline material. Further work has since demonstrated that previous rations were low in a number of factors, the most outstanding of which was pantothenic acid. A basal ration developed in this laboratory (Conger and Elvehjem, '41) has been shown to be adequate in all respects except for vitamin B_6. When the crystalline vitamin is added at levels of 2–6 µg. per day, the growth response is proportional to the amount of crystalline material.

<div align="center">EXPERIMENTAL</div>

The method of assay used is essentially that of Conger and Elvehjem ('41). The basal ration has the following percentage composition: purified casein [2] 18; sucrose 75; salts IV [3] 4; corn oil [4] 3. Each 100 gm. of this mixture contained the following: thiamine 200 µg.; riboflavin 300 µg.; nicotinic acid 2.5 mg.; choline 30 mg.; and calcium pantothenate [5] 1 mg.; fullers earth filtrate of liver conc. 1:20 ≅ 4% original liver powder; and haliver oil 2 drops weekly.

The fullers earth filtrate was prepared by dissolving 100 gm. of liver concentrate powder 1:20 in 100 ml. of distilled water and shaking with 500 ml. of butanol in a mechanical

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[2] Labco casein, Borden and Company, New York City.

[3] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935). Salt mixture with $MnSO_4$: $4 H_2O$ increased from 0.7 to 10 gm. per kilogram of salt mixture.

[4] Corn oil is added to the remainder of basal ration only in amounts sufficient to last 1–2 weeks and rations are kept at low temperature in the dark to prevent rancidity.

[5] Merck's.

shaker for 2 hours. After the two layers separated, the butanol layer was removed and 500 ml. more butanol added. This extraction with butanol was continued until five extractions had been made. When anhydrous butanol was used, it was found necessary to add approximately 25 ml. of water following the second or third extraction to prevent the water layer from becoming too viscous for effective extraction. The combined extracts were concentrated under reduced pressure to a syrup at which point water was added and concentration continued to remove the last traces of butanol. The residue was taken up in 100 ml. of distilled water and adjusted to pH 1–2. Five grams of English fullers earth was then added to adsorb out the vitamin B_6. After shaking for 20 minutes the fullers earth was removed by filtration through a thin pad of coarse filter cel. The pad was washed with 10–15 ml. of N/10 HCl. The adsorption on fullers earth was repeated four more times using 5 gm. portions of the fullers earth each time. The combined filtrate and washings were neutralized to pH 7 with NaOH and concentrated under reduced pressure. The concentrated filtrate was dried on the casein of the ration at a level equivalent to 4% of the original liver concentrate. Water solutions of the vitamins were spread on the casein and dried in a current of warm air at 50°C. The salts and sucrose were then added and the entire ration was finely ground.

Male albino rats weighing 35–40 gm. at 21 days of age were used throughout the assays. The rats were placed in individual cages and fed the basal ration for a 2-week depletion period. An average gain of 10 gm. during the first week and 7 gm. during the second week of the depletion period was obtained. The average gain of control rats continued on the basal ration was 5 gm. per week for a 5-week period. At the end of the 2-week depletion period the standard controls were fed the basal ration to which crystalline vitamin B_6 had been added at levels of 50, 75 and 100 μg. per 100 grams of ration. The rats on the standard amounts of pyridoxine were included in each series of assays to eliminate possible individual differences. More than thirty rats on the crystalline pyridoxine showed

remarkable uniformity in growth responses to the three levels of the vitamin throughout the assay period of 5 weeks. Conger and Elvehjem ('41) have demonstrated that the ration is complete in all factors except vitamin B_6 and that growth is the best criterion for measuring the pyridoxine potency.

The meats, which were prepared for assay according to methods previously described by Mickelsen, Waisman and Elvehjem ('39 a) were mixed in the basal ration. Four to eight rats were used to obtain the pyridoxine values for a particular sample. The rats were weighed and examined at weekly intervals. No acrodynia or other gross symptoms were observed in the control rats or in the animals receiving the supplement during the assay period. At the end of a 5-week assay period the vitamin B_6 content of the meats was calculated by comparing the growth of the rats on the meat supplement with the growth of the rats receiving crystalline pyridoxine (vitamin B_6 hydrochloride). Preliminary work showed that when calculations were based on the percentage of meat in the ration, more consistent results were obtained than when food consumption records were used.

<center>RESULTS</center>

The results of our bioassays are shown in table 1. The values are expressed in micrograms per gram of fresh and dried meat. Kidney is a good source of vitamin B_6. Two samples of beef kidney and two samples of pork kidney showed approximately the same content of the vitamin. With the exception of the beef liver sample, livers of the various species contained less than 15 µg. per gram of dried tissue. Lamb liver was slightly higher than pork liver and veal liver. Muscle was a moderately good source of pyridoxine. Three samples of pork ham contained 20–28 µg. per gram dry weight. Three samples of pork loin showed a vitamin B_6 content of 14–19 µg. per gram of dried loin. Beef muscle was only slightly lower than veal muscle. Lamb muscle fell in the same range. Pork and beef heart had a somewhat lower vitamin B_6 potency than other muscle meats. A single sample of dark chicken showed

TABLE 1

Pyridoxine content of meat and meat products

SAMPLE	SAMPLE NUMBER	VITAMIN B₆ HYDROCHLORIDE (µG. PER GRAM)	
		Dry	Fresh
Beef brain	77	5.3	—
Beef heart	73	11.2	2.4
Beef kidney	126	21.3	4.3
Beef kidney	81	19.5	4.4
Beef kidney (stewed)	112	14.4	—
Beef liver	131	25.0	7.3
Beef lung	138	3.5	0.7
Beef muscle	128	16.2	3.8
Beef muscle	105	14.5	4.0
Beef muscle (fried)	106	12.0	—
Beef pancreas	113	7.5	2.0
Beef spleen	130	5.7	1.2
Beef tongue	82	4.2	1.2
Chicken (dark)	66	< 8	< 2
Lamb muscle	80	11.8	3.0
Lamb liver	96	12.6	3.7
Pork ham (dried)	95	24.6	6.0
Pork ham (dried)	124	28.2	6.7
Pork ham (dried)	139	21.7	5.5
Pork ham (fresh)	140	23.5	5.9
Pork ham (fried)	141	15.7	—
Pork ham (roast)	142	12.1	—
Pork ham (fried)	52	17.6	—
Pork ham (boiled)	115	10.6	—
Pork ham (smoked)	116	9.8	2.9
Pork ham (tender)	72	7.7	2.4
Pork ham (tender)	119	8.8	2.9
Pork ham (tender)	117	11.0	3.8
Pork loin	125	18.8	5.1
Pork loin	89	19.5	6.5
Pork loin	36	14.5	4.5
Pork loin (roasted)	94	11.5	—
Pork kidney	83	18.1	4.0
Pork kidney	137	34.8	7.0
Pork heart	104	15.6	3.5
Pork liver	86	9.9	3.2
Pork liver	122	10.3	3.3
Veal liver	97	10.3	3.0
Veal muscle	129	16.2	4.0
Veal muscle	134	19.4	4.2
Veal muscle	103	16.3	4.4
Frozen fillet of cod	135	19.6	3.4
Frozen salmon steak	136	21.2	5.9
Yellow corn		4.8	—
Wheat germ		15.9	—
Winter milk		—	1.3

a low vitamin B_6 content, although the levels fed were too low to ascertain exactly how much pyridoxine was present. Beef tongue, beef spleen, beef pancreas and beef brain were in the range of the lower potencies. Beef lung was the poorest source of the vitamin. Since fish muscle had been reported to be a good source of the "anti-acrodynia" factor by György ('35), Wilson and Roy ('38), and Lunde and Kringstad ('38), we assayed samples of cod and salmon. The samples were obtained in the frozen state and dried in the usual manner. The results indicated that fish muscle is indeed a good source of vitamin B_6 containing about the same as mammalian muscle.

Determinations made on processed hams showed a marked reduction in the pyridoxine potency as a result of the processing. The boiled ham sample contained approximately half that of pork ham, and smoked ham showed a similar decrease. Three samples of tender ham showed a loss of about 60% of the vitamin B_6 as a result of the tenderizing process. Stewed beef kidney and fried pork retained two-thirds of their original potencies. Pork loin showed a reduction of almost half in the roasting process. A single fresh pork ham was divided into four portions in order to further ascertain the loss of the vitamin during household cooking. These portions were fed fresh (sample 140), dried (sample 139), fried (sample 141) and roasted (sample 142). The results obtained by assaying these samples showed that the drying process resulted in no significant loss of vitamin B_6 potency, while frying resulted in about 30% loss and roasting in 45% loss. A slight loss was also noted during the frying of beef muscle as shown by the vitamin B_6 content of the parallel samples 105 and 106 which were from the same animal. The loss of vitamin B_6 during various cooking processes is probably the result of leaching since this vitamin is known to be heat stable and very soluble in water when in the free form.

A single sample of yellow corn contained 4.8 μg. per gram, a value less than any meat assayed except beef tongue and beef lung. This is contrary to the early report of Copping

('36) which stated that corn was a good source of vitamin B_6. Wheat germ was found to contain almost as much pyridoxine as most muscle meats. Winter milk showed a potency of 1.3 µg. per milliliter. It is difficult to compare the values we obtained for the pyridoxine content of meats with the few values reported for the "antiacrodynia" potency of meats reported by György ('35) and Schneider, Ascham, Platz and Steenbock ('39). As yet the only report of the vitamin B_6 content of meats using a chemical method was one in which Swaminathan ('40) reported 13.4 µg. per gram for fresh sheep liver and 4.5 µg. per gram for fresh sheep muscle. The value for muscle is about the same as we obtained, 3.0 µg. per gram fresh, but his value for liver is considerably higher than ours. There is good agreement between our value for milk of 1.3 µg. per milliliter and Swaminathan's of 1.7 µg. per gram.

A previous report (Mickelsen, Waisman and Elvehjem, '39 a) has demonstrated that pork muscle is a better source of thiamine than liver, whereas the assays for riboflavin (Mickelsen, Waisman and Elvehjem, '39 b), pantothenic acid (Waisman, Mickelsen and Elvehjem, '39) and nicotinic acid (Waisman, Mickelsen, McKibbin and Elvehjem, '40) have shown that liver is invariably superior to muscle tissue as a source of these factors. The assays reported here, however, indicate that muscle tissue must be considered a better source of vitamin B_6 than liver. It should be emphasized that considerable variation in the B_6 content of a given tissue may be encountered if several samples are examined. However, assay of a few samples of each type of tissue gives values which allow a comparison of one meat with another and with other foodstuffs.

Although there are no accurate figures available on the human requirement for pyridoxine, it may be possible to approximate the amount necessary from the human requirements of other vitamins and comparative growth experiments with rats using the same vitamins. It has been shown by work in this laboratory (Arnold and Elvehjem, '38; Waisman,

Henderson and Elvehjem, '40) that 100 µg. of thiamine and 200 µg. of riboflavin per 100 gm. of diet will produce optimum growth in the rat. The level of pyridoxine necessary to give maximum growth in the rat lies between these levels of the other vitamins, but closer to that of thiamine. Since the daily human requirement for thiamine is about 2 mg. per day (Cowgill, '39; Sherman and Lanford, '39), it appears that the daily requirement of pyridoxine would also be about 2 mg. Using this figure, meat is able to furnish a large part of this requirement since the average vitamin B_6 content of muscle tissue is 5–6 µg. per gram of the fresh meat. This means that 300–400 gm. of fresh muscle meat is necessary to supply the daily need of pyridoxine.

SUMMARY

1. The pyridoxine content of meat and meat products has been determined by means of a rat growth method.

2. Kidney and muscle were the richest sources containing 20–30 µg. per gram while heart and liver were somewhat lower with 10–15 µg. per gram; spleen, pancreas, brain and lung were poor sources containing less than 8 µg. per gram on the dry basis.

3. The fried meat samples showed least destruction, but roasting and stewing caused losses of from 20 to 50%. Commercial processing of various meat samples resulted in similar losses.

4. Some samples of fish muscle, milk, and cereals were also assayed.

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SODIUM AND POTASSIUM SALTS IN THE TREATMENT OF EXPERIMENTAL DIABETES AND DIABETES MELLITUS [1]

SOLOMON STROUSE, FLORENCE BUEL, RAYMOND KAY
AND DOUGLAS DRURY

*Departments of Medicine and Physiology, University of Southern California,
and the medical service of the Los Angeles County Hospital*

ONE FIGURE

The relation of sodium and potassium salts to carbohydrate metabolism has been sporadically investigated for a number of years. Beard ('18) studied the effect of unrestricted salt intake on diabetic patients, especially from the point of view of possible kidney damage. In the course of his paper, he mentions that only one patient on high sodium chloride, and one on high sodium bicarbonate showed increased sugar tolerance. He observed that his studies allowed no deductions concerning the utilization of carbohydrates during the period of high sodium chloride intake. Glass and Beiless ('30) reported beneficial effect of intravenous injections of hypertonic sodium chloride solution on the blood sugar in diabetes.

The subject received new attention when McQuarrie, Thompson and Anderson ('36) gave large doses of salt (60 to 90 gm. daily) to a series of diabetic children. Four patients, 13 to 15 years old, were intensively studied in a metabolism ward. A basal diet with known very low potassium and sodium values was used throughout the experiments. These authors claimed

that following the ingestion of these large doses of sodium chloride, blood pressure rose, glycosuria lessened, fasting blood sugar levels were lower, the R.Q. increased, and insulin reactions were more frequent. The blood pressure and the glycosuria returned to previous level when the sodium chloride was stopped. Potassium acted in just the opposite manner, and when sodium and potassium were balanced in the McQuarrie basal diet, no noticeable change occurred. MacLean ('35) reported similar results in a diabetic patient at the Mayo Clinic. Sanstead ('36) gave doses of sodium chloride varying from 10 to 90 gm. to eight male adult diabetics, and in all his patients he found a lowering of blood sugar. A reduction of insulin or an increase in diet was made possible by this high ingestion of sodium chloride. His protocols do not indicate the effect on glucose excretion, nor is there any reference to the ratio of sodium and potassium.

EXPERIMENTS ON PATIENTS

Our first experiments were carried out in a series of twelve patients in the diabetic ward of the Los Angeles County Hospital without extensive preliminary metabolism studies. These patients were stabilized and without changing the diet were then given doses of sodium and potassium chloride varying from 20 to 30 gm. daily. We found that larger doses usually could not be employed without causing nausea. Blood sugar, glycosuria, and blood pressure were carefully followed. Critical analysis of the data from these experiments failed to reveal any constant or marked change in the patient's status which could definitely be attributed to the change in therapy. Occasionally minor changes in glucose excretion were noted after the administration of sodium chloride or of potassium chloride, but these changes were not constantly in the same direction. For example, in any one patient carried through several experimental periods the administration of sodium chloride was followed at one time by decreased glucose excretion and later by increased excretion. The same variability in results was

noted in the experiments with potassium chloride. An illustrative protocol of the experiments on human subjects is presented in table 1.

TABLE 1

Effect of increasing sodium intake on glucose excretion in a diabetic patient [1]

| DATE | INTAKE | | | URINE | | | BLOOD | |
	Carbo-hydrates	Sodium	Glucose	Sodium	Potas-sium	Sugar	Serum sodium	Serum potassium
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*mg./100 cc.*	*mg./100 cc.*	*mg /100 cc.*
5–24	173	0.921	19.09	1.405	0.842			
5–25	173	0.921	37.32	0.890	0.841			
5–26	173	0.921	43.15	1.44	1.03	290	329	18
			Sodium period					
5–27	179	8.771	39.12					
5–28	179	8.771	36.70	3.03	0.995			
5–29	182	12.721	42.2	9.70	1.51			
5–30	182	12.721	32.21	8.77	1.72			
5–31	182	12.721	41.35	8.16	1.62			
6– 1	182					238	332	19

[1] It should be noted that the "en seal" tablets of sodium chloride used in this experiment contained a small amount of carbohydrate which accounts for the differences in carbohydrate intake.

The data here presented were obtained from one patient, a 14 year old girl who was placed on the McQuarrie diet for a period of 11 days during which time the insulin was adjusted to allow a fairly constant glucose excretion. The diet contained 173 gm. carbohydrate, 70 gm. protein, 89 gm. fat, 0.921 gm. sodium, 1.963 gm. potassium. For the first 2 days 20 gm. of sodium chloride were given daily and 30 gm. daily thereafter. Insulin was constant for the experimental period at 10-0-10 units regular insulin. Certainly in this patient no effect of 20 or 30 gm. of sodium chloride on glucose excretion could be demonstrated.

At this stage it was apparent that our investigations were not demonstrating the uniform pattern suggested by previous authors. Varying and inconclusive results provided considerable contrast to the almost perfect score of McQuarrie et al. and of Sanstead. It seemed then logical to assume that if sodium and potassium were important in diabetic therapy,

some other factor than the mere addition of either ion to the diet was necessary to explain the action.

Experiments were projected on various animal species. Our preliminary tests on rats indicated that no definite change in sugar excretion followed alterations in potassium and sodium intake, and for this reason no further studies were undertaken. Technical difficulties caused us to eliminate the rabbit. The goat was in many respects a satisfactory experimental subject, and we shall later present the protocols on some of our investigations on this animal.

EXPERIMENTS ON DOGS

Our experiences, like those of other students of carbohydrate metabolism, have indicated that the dog is an excellent subject for experiments of this nature. Female dogs were depancreatized and given enough food and insulin to keep the weight of the animal constant and still have a definite but not unduly large glycosuria. Then the animals were not used until at least a month after operation and only at a time when they had completely recovered and were in good condition. After the control period an experimental period of 3 days was run under identical conditions except for the addition of sodium or potassium chloride to the diet. This time period was soon found to be inadequate and was increased to 5 days and still later to even longer periods. The dogs' food was divided into two equal portions and fed at 8 A.M. and 5 P.M. daily. Protamine Zinc Insulin was injected subcutaneously immediately after each feeding. The dogs usually ate their food within a few minutes, but whenever it was necessary to coax the dogs to eat, the food was always consumed within ½ hour. The animals were kept in metabolism cages which were cleaned daily. Urine collections for a 24-hour period were made at 8 A.M. Venous blood for blood sugar determinations was taken immediately before feeding. Blood for serum determinations of sodium and potassium was always taken before the 5 P.M. feeding. The methods used for chemical analyses were as follows: Blood sugar, Folin-Wu; urine sugar, Benedict's

quantitative; urine and serum sodium, Hoffman adapted by Chaney; urine and serum potassium, Brown, Robinson, and Browning adapted by Chaney.

In the first experiment a diabetic dog subsisted on a constant diet of meat, pancreas and sugar. This contained 100 gm. of carbohydrate and between 1.3 and 2.2 gm. of potassium per day. This animal was observed during seven 5-day periods, arranged so that the control periods alternated with the experimental. In two of the experimental periods 2.3 gm. extra sodium were given, and in one period 4 gm. extra potassium. In both instances the salts given were chlorides. We found that the animal excreted an average of 43 gm. of glucose per day during the first control period, but during all the remaining periods the average daily excretion varied between 22 gm. and 25 gm. In other words, the glucose excretion remained practically constant during the last six periods, and suggests that the fall from the initial period was the result of spontaneous improvement.

TABLE 2

Effects on glucose excretion of increases of potassium and of sodium intake in the diabetic dog. The basal diet was different in parts A and B

| | A | | | | | B | | |
	I Control	II Potassium	III Control	IV Sodium	V Control	VI Sodium	VII Control	VIII Sodium
No. days	16	9	33	5	15	8	6	6
Average glucose output (gm.)	11.08	25.07	14.31	7.64	18.39	23.10	32.50	26.60
Sodium intake (gm.)	0.611	0.611	0.611	4.84	0.544	3.696	0.044	3.190
Average sodium output (gm.)	0.641	0.650	0.618	4.190	0.511	4.777	0.032	2.98
Potassium intake (gm.)	0.900	4.030	0.900	0.900	0.561	0.561	0.561	0.561
Average potassium output (gm.)	0.800	3.74	0.739	0.316	0.754	0.470	0.310	0.736
Average of daily blood sugars (mg./100 cc.)	250 a.m. 183 p.m.	560 a.m. 275 p.m.	500 a.m. 260 p.m.	520 a.m. 260 p.m.	420 a.m. 250 p.m.	416 a.m. 269 p.m.	400 a.m. 242 p.m.	

An attempt was then made to duplicate the results obtained by McQuarrie et al., using the diet of milk, eggs and sucrose as employed by them in their studies on children. Choline hydrochloride (100 mg. daily) was also used in the dog experiments to replace pancreas. This is necessary to prevent fatty degeneration of the liver.

As will be seen by examination of table 2A, the average daily glycosuria increased during the high potassium period and decreased during the high sodium period. Further examination of these apparent results, however, is deemed necessary. The increase in glucose output during the potassium period over that of the control period is certainly marked. It was considered advisable to extend the subsequent control period to 33 days because during this time, despite a rigidly constant regimen, the glycosuria was steadily decreasing. We have charted successive subdivisions of this period, and figure 1 shows this definite trend toward diminishing glycosuria. The chart illustrates the spontaneous changes which may occur in the status of diabetic animals. During this period had we in
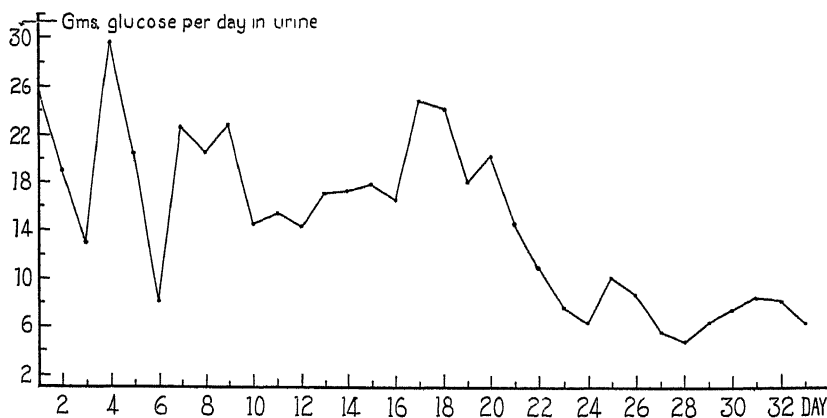
Fig. 1   Shows variations in glucose excretion of a diabetic dog under constant conditions. Besides the ordinary day-to-day fluctuations there is to be noted a definite improvement (beginning on the twentieth day) in the diabetic condition during the later days. This illustrates the ''spontaneous changes'' which may occur in diabetic animals despite the maintenance of rigidly constant conditions.

any way changed the experimental conditions, we might easily have been misled into drawing erroneous conclusions. During the last 13 days of this control period, the dog was excreting only an average of 6.63 gm. of glucose daily. Then, upon the addition of sodium chloride to the diet (to give an average intake of 4.84 gm. of sodium daily) the glycosuria actually increased over the last few days of the preceding control period.

Because of intestinal disturbances, resulting from the high salt administration, it was now necessary to adopt a new diet. In order to keep the potassium intake at a minimum, a diet of pancreas (which is very low in potassium) and sucrose was substituted. As will be noticed upon examination of this experiment in table 2B, there are no consistent results. In the 29 days comprising the first three periods a gradual increase in glycosuria occurs even though sodium chloride has been given for 8 days between two control periods. The final period of increased sodium chloride intake is associated with a slightly diminished output of glucose. We do not see any definite indications of a sodium effect in this experiment.

A second depancreatized dog gave equally negative results when carried through the same type of experiment. During a period of 8 days, with a constant diet of pancreas and sucrose containing carbohydrate 80 gm., sodium 0.044 gm., and potassium 0.561 gm. daily, the average daily glucose excretion was 2.13 gm. During the next period of 19 days the carbohydrate and sodium were kept the same and the potassium was increased to 3.7 gm. per day. The average sugar excretion remained practically the same, 1.98 gm. During the next control period (5 days) the carbohydrate intake was increased to 150 gm. per day and the sodium to 0.544 gm. per day, with the potassium 0.561 gm. per day. The average sugar excretion was 26.2 gm. During the fourth period (7 days) with the carbohydrate and potassium the same, the sodium intake was increased to 3.96 gm. per day, and the average sugar excretion was 12.44 gm. per day.

EXPERIMENTS ON A GOAT

The depancreatized goat is able to utilize approximately 80% of the carbohydrate ingested without insulin, and at the same time maintain its weight. The diet consisted of sucrose and alfalfa. Weighed amounts of alfalfa were given the goat daily, and the part not eaten was weighed. Because the same amount of food was not eaten each day we have substituted the term "per cent of glucose utilized" for the phrase "glucose excretion" used in previous tables. The sugar solution, or the sugar solution plus the added sodium or potassium chloride, was taken at the same time daily, 8.30 A.M., but the alfalfa was eaten throughout the day. All blood chemical determinations were made on venous blood drawn between 3.30 P.M. and 4.00 P.M. Any attempt to substitute a food lower in potassium for the alfalfa resulted in the death of the animal. The potassium content of alfalfa is about 2%. The protocols show no marked effect on the carbohydrate utilization following the addition of sodium or potassium to the diet. The results of an experiment on a diabetic goat are found in table 3. This experiment on the goat may be of importance because, in the presence of considerable glycosuria, variations of sodium and of potassium intake were followed by definite changes of excretion of these ions, yet were without any significant influence on the excretion of sugar or on the blood sugar.

TABLE 3

Effects on glucose excretion of increases of potassium and of sodium intake in the diabetic goat

	I Control	II Sodium	III Control	IV Potassium	V Control	VI Sodium
No. days	4	5	5	5	12	8
Average glucose output (gm.)	55.9	64.9	57.5	47.1	67.3	57.0
Glucose utilization (%)	79.81	77.2	77.8	81.2	74.2	79.6
Sodium intake (gm.)	0.76	2.51	0.62	0.55	0.66	3.00
Average sodium output (gm.)	0.530	1.98	0.40	0.54	0.178	2.38
Potassium intake (gm.)	10.00	9.30	7.80	10.80	8.50	8.80
Average potassium output (gm.)	13.4	10.2	10.5		11.02	11.08
Average of daily blood sugars (mg./100 cc.)	160 p m.	189 p.m.	250 p m.	267 p.m.	252 p.m.	259 p.m.

We studied the diabetic goat during another experiment lasting 27 days during which time the animal was fed as before but given 8 units of insulin per day. The time was divided into four periods consisting of two control periods interposed between a high sodium and a high potassium period. Here, as before, variations of sodium and of potassium intake were followed by corresponding changes of excretion of these ions without any change in the average daily urinary glucose or in the "per cent utilization of carbohydrate".

SUMMARY

Our experiments on human subjects indicate that there is no constant response to the ingestion of sodium chloride which warrants a conclusion as to its therapeutic value in diabetes mellitus. Limited experiments with potassium likewise gave inconclusive results. In some patients an apparent change in carbohydrate metabolism following the ingestion of sodium or of potassium is suggested, but this phenomenon is not sufficiently constant to justify any sound conclusions.

Studies on diabetic animals definitely show that ingestion of sodium chloride or of potassium chloride may be followed by changes in sugar excretion of such magnitude as to suggest causal relationship. However, these changes are not constant. In the same animal, under experimental conditions identical as far as can be determined, the ingestion of sodium chloride is followed at one time by diminished glycosuria and at another time by increased glycosuria. Contrasting experiments on two dogs, the same paradoxical results are noted. Detailed analyses of our tables indicate that in some instances the results are more apparent than real and may actually represent coincidental changes in status.

Our results do not entirely agree with those reported by McQuarrie, Thompson and Anderson. Differences in conditions of the experiments may to some extent account for this. Their experiments were conducted on children to whom they gave very large doses (60 to 90 gm.) of salt; most of our work was done on adult diabetics or on the depancreatized animal,

and we were unable to employ the large salt dosage used by McQuarrie et al., without producing nausea, vomiting, or diarrhea. The most convincing experiment published by them concerned a salt craver who showed definite decrease in sugar output when sodium chloride was given. In no other case are their results as conclusive: the change in sugar output following the ingestion of sodium chloride or of potassium chloride suggests causal relationship, but may also be interpreted as a change in status of the patient. The possibilities of such changes influencing conclusions from therapeutic experiments in diabetes are explored and extended in our discussion of figure 1.

The experiments on the goat seem of particular interest in that large changes in mineral equilibrium were produced by our experiments without demonstrable corresponding changes in sugar metabolism.

It is probable that changes in cellular and tissue metabolism not elucidated by our protocols may explain the results. Our human and animal studies suggest one conclusion: changes in carbohydrate metabolism which follow ingestion of sodium or potassium chloride cannot be explained as a result of the mere addition of these salts to the dietary intake.

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REQUIREMENT OF THE MOUSE FOR PANTOTHENIC ACID AND FOR A NEW FACTOR OF THE VITAMIN B COMPLEX [1]

JOSEPH G. SANDZA AND LEOPOLD R. CERECEDO

Department of Chemistry, Fordham University, New York

TWO FIGURES

Pantothenic acid has been shown to be required by the chick (Jukes, '39; Woolley et al., '39), the rat (Hitchings and Subbarow, '39; El-Sadr et al., '39; Oleson et al., '39), the dog (McKibbin et al., '40), and by man (Spies et al., '40). The instability of pantothenic acid to hot alkali has become an important factor in the investigation of this vitamin. Pennington, Snell and Williams ('40) have inactivated pantothenic acid in a yeast extract by autoclaving for 30 minutes with 0.5 N sodium hydroxide at 15 pounds pressure. McKibbin and his co-workers ('40) were able to destroy pantothenic acid in a liver extract by heating at 87–93° C. for 2 hours in the presence of 0.2 N potassium hydroxide.

In this paper, data will be presented which indicate that the mouse requires pantothenic acid and one or more factors contained in the alkali-stable portion of the filtrate fraction of the vitamin B complex.

EXPERIMENTAL

The diets used had the following percentage composition: purified casein, 30; Osborne and Mendel salt mixture, 7; sucrose, 55; hydrogenated cottonseed oil,[2] 5; cod liver oil, 3.

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[1] Presented in part at the meeting of the American Chemical Society at Detroit, September, 1940.

[2] Crisco.

Ten milligrams of riboflavin, 5 mg. of thiamine chloride, and 5 mg. of vitamin B_6 hydrochloride were added per kilo of diet. To this basal diet (diet B) alkali-treated liver extract was added, in an amount equivalent to 50 gm. of liver extract [3] per kilo of diet (diet B-L).

The alkali treatment of the liver consisted in heating on the steam bath for 1 hour, with occasional stirring, a solution of 100 gm. of 92% alcohol soluble liver extract in 500 cc. of water, to which 20 gm. of sodium hydroxide (to make 1 N) had been added. The solution was allowed to cool, and made just acid to litmus with sulfuric acid.

Albino weanling mice, 21–25 days old and bred in our laboratory, were placed in individual wire-mesh cages and fed a diet free from water-soluble vitamins (diet-B without addition of vitamins), for a period of 5 days. They were then fed the experimental diets. Those animals receiving the pantothenic acid supplement were injected subcutaneously with 40 micrograms daily (6 days per week) of d-calcium pantothenate [4] in water solution.

The pantothenic acid requirement was determined by injecting varying amounts of d-calcium pantothenate daily (6 days per week) into each of five litter-mate groups, each group consisting of eight animals. They were given diet B. The data presented in figure 1 show that under the experimental conditions present the mouse requires about 30 micrograms of d-calcium pantothenate daily.

Mice receiving the unsupplemented basal diet (diet B) grew for a short time, after which they exhibited only maintenance (fig. 2). After 20–30 days, they showed symptoms of filtrate factor deficiency, characterized by loss of hair in one or more areas of the body, which turned into complete alopecia in the later stages. They also developed a serous exudate around the eyes, spinal curvature, and a kicking twitch of the hind legs. The animals finally lost weight and died on about the

[3] Supplied by the Wilson Laboratories, Chicago, Ill.

[4] The d-calcium pantothenate was generously supplied by Merck and Company, Rahway, N. J.

fifty-fifth day. A diet containing yeast or whole liver extract protected completely against this deficiency.

When the basal diet (diet B) was supplemented with 40 micrograms daily (6 days per week) of d-calcium pantothenate, the animals grew at a rapid rate (fig. 2), and showed no signs
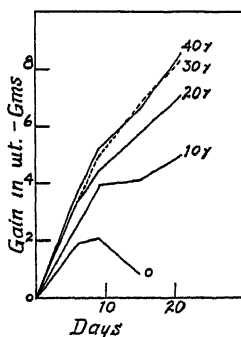
Fig. 1   Growth of mice receiving increasing amounts of d-calcium pantothenate daily (6 days per week). The animals were fed diet B. Each curve represents the average for eight mice.
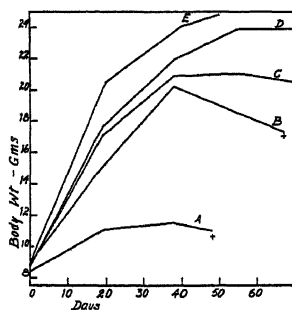
Fig. 2   Average growth curves of mice receiving various fractions of the vitamin B complex. Curve A shows the growth of animals receiving only diet B, and curve B that of animals receiving diet B-L. Curve C shows the growth response of mice receiving diet B plus 40 micrograms daily (6 days per week) of d-calcium pantothenate, and curve D that of mice receiving diet B-L plus the same amount of d-calcium pantothenate. Each curve represents the average for eight animals. For comparison curve E showing the average growth of mice fed upon our stock diet has been included. The difference between the growth rate of the control animals (curve A) and that of the control animals shown in figure 1 is due to individual variations, and appears to depend on the reserves of the missing factors in the young mice.

The (+) indicates the time at which the first animal died.

of deficiency until about the fortieth day. At this time the coat became shaggy, and the animals stopped growing.

Mice receiving the alkali-treated liver extract in addition to thiamine, riboflavin, and vitamin B_6 (diet B-L), did not grow as well as animals on diet B plus pantothenic acid, and showed signs of deficiency at an earlier date. By the eighty-sixth day, one-half of the animals were dead.

When diet B-L was supplemented with calcium pantothenate, the animals grew at a rate which was close to normal, and in the majority of cases appeared in good condition. Some of these animals, however, presented an unclean appearance similar to that shown in the beginning stages of filtrate factor deficiency. The reason for this has not yet been ascertained.

These experiments have been repeated, using the butanol-soluble portion of the 92% soluble liver extract [5] as a source of the filtrate fraction of the vitamin B complex, with similar results.

These observations indicate that in addition to thiamine, riboflavin, and vitamin B_6, the mouse requires pantothenic acid and an alkali-stable member of the filtrate fraction of the vitamin B complex. Our findings are in agreement with those reported for the rat by Dimick and Lepp ('40) and by Unna ('40). This unknown factor may be identical with Elvehjem's factor W. Experiments conducted in our laboratory [6] have shown that mice which have ceased to gain in weight after being kept on a diet containing thiamine, riboflavin, nicotinic acid, and vitamin B_6, will increase in weight at a rate close to normal if they are given a daily dose of a factor W concentrate (prepared according to Black et al., '40). Whether this response is due to the pantothenic acid present in the concentrate alone, or to the combined effects of pantothenic acid and some unknown factor, are points which still require investigation.

[5] Prepared according to the initial step in the preparation of factor W concentrate (Black et al., '40).

[6] Unpublished data.

DISCUSSION

The possibility of the existence of a second alkali-labile factor is not excluded by these experiments. Animals receiving untreated liver extract show a better growth and appear to be in better condition than those receiving alkali-treated liver extract plus pantothenic acid, and they behave similarly in all respects to animals kept on our stock ration. Whether this difference is due to the presence in liver extract of an alkali-labile factor distinct from pantothenic acid, or to some other cause, cannot be decided from these experiments. McKibbin, Black and Elvehjem ('40) have recently reported a series of experiments on dogs, using an inactivated liver preparation similar to that used in our experiments. On the evidence of these experiments they suggest the existence of an alkali-labile factor, distinct from pantothenic acid, which is required by the dog.

Woolley ('40 a, '40 b) has reported that inositol, or the salt of its phosphoric ester, phytin, is required by the mouse as a growth and "anti-alopecia" factor. According to his experiments, mice receiving all the known crystalline components of the vitamin B complex in addition to yeast extract, ceased to grow in about 4 weeks, and developed marked loss of hair within 6 weeks. Unless the new factor was given, these animals died within 2–3 weeks from the onset of symptoms.

In our experiments, no case of marked alopecia has been observed among fifteen mice which have received only thiamine, riboflavin, vitamin B_6 and pantothenic acid, and these animals are still alive after 140 to 185 days on the experiment. Our basal diet, however, differs from that used by Woolley in that it contains more protein, less sucrose, more fat, no yeast extract, and especially a larger amount of pantothenic acid.

Norris and Hauschildt ('40) have reported that mice require a water-soluble factor other than thiamine, nicotinic acid, vitamin B_6, riboflavin and the "filtrate factor." In the absence of this factor from the diet, mice were found to develop skin lesions similar to those which we have observed

in filtrate factor deficiency, but which, according to our observations, are prevented in every case, and can be cured, by pantothenic acid. Here again, our diets differ in composition from that used by these workers.

To what extent the discrepancy between our results and those reported by Woolley, and by Norris and Hauschildt, can be explained by the differences in the rations used or in other experimental conditions, remains to be determined.

Our findings indicate that for mice, as has been reported for rats, four components of the vitamin B complex, namely, thiamine, riboflavin, vitamin B_6, and pantothenic acid, provide an almost adequate supplement. The deficiency of the unknown factor shows itself mainly in the suboptimal growth, but the animals survive for more than 6 months.

SUMMARY

It has been shown that besides thiamine, riboflavin, and vitamin B_6, at least two additional factors are necessary for normal growth in the mouse. One of these is pantothenic acid. Under the conditions present in our experiments, the daily requirement of the mouse for this factor is about 30 micrograms in terms of d-calcium pantothenate. The second of these is alkali-stable and is present in liver extract.

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IRON METABOLISM IN HUMAN SUBJECTS ON DAILY INTAKES OF LESS THAN 5 MILLIGRAMS [1]

RUTH M. LEVERTON [2]

Human Nutrition Laboratory, Department of Home Economics,
University of Nebraska, Lincoln

TWO FIGURES

The requirement of the human for iron is undisputed but the amount required is controversial. The pendulum of recommendations for daily intakes has swung from a high of 17 mg. (Leverton and Roberts, '37) to a low of 5 mg. (Farrer and Goldhamer, '35) but has rested for the most part between 12 and 15 mg. of iron (Sherman, '32, '37).

Recent developments in the field of research have challenged any interpretation of requirements which is based upon determining the relation of the intake to the excretion of the metal and using this relation as a measure of the body's need for iron.

McCance and Widdowson ('38) have presented substantial evidence for their theory that the gastrointestinal tract has no ability to regulate by excretion the amount of iron in the body. This means that the iron found in the feces is that which has not been absorbed rather than iron which has been absorbed, utilized, and re-excreted. It follows then that the iron excreted in the feces is not a criterion of the body's utilization or need but rather a reflection of its absorption, and many factors other than need may influence absorption.

In addition to this changed viewpoint much has been done in the last few years to direct attention to the great theoretical and practical significance of serum or plasma iron. Several investigators, Moore, Doan and Arrowsmith ('37), Heilmeyer and Plotner ('37) and Baer ('38) have shown that serum iron is of metabolic importance in the mammalian organism because it serves as a medium for iron transportation. Of particular value to the study of iron metabolism is the evidence presented by Moore et al. ('39) and Hahn et al. ('38) to show that the amount of iron being absorbed from the intestinal tract is indicated by increases in the plasma or serum iron.

With these facts in mind, namely, that the intestine probably does not excrete iron and iron excretion, therefore, is not a measure of utilization, and that changes in serum iron values may indicate iron absorption, the present study was planned for three, and later four college girls. The first objective was to follow the fecal excretion of iron on a diet which contained as little iron as possible and yet could be considered adequate in every other respect. It was of particular interest to see if the fecal excretion of iron would ever significantly exceed the intake, regardless of how low the intake became. The second objective was to follow the serum or transport iron values on this low iron intake in an effort to study the relationship of the level of intake and excretion to endogenous iron metabolism. Later a third objective developed, namely, to study the effect of substituting meat for the milk in the basal diet.

PROCEDURE

The plan of the study was to follow, over a period of several months, the excretion of iron by young women on a constant diet optimum in all known dietary essentials except that it contained less than 5 mg. of iron, and to make closely associated blood tests which might reflect changes in the iron content of the body as a result of such a low iron intake.

The subjects were four healthy college girls who will be referred to as subjects A, B, C and D. Subject A was 21,

subjects B and C were 22, and subject D was 28 years old at the beginning of the study.

Subjects A, B and C were on the metabolism regimen continuously for 94 days from September 19 to December 23, 1939. On January 4, 1940, subjects A and B returned to the regimen, subject A for 90 days until April 8th, and subject B for 150 days until June 2nd. Subject C was studied again for 25 days in March and subject D for 75 days from the middle of March until June. This totals 622 days of study on four individuals.

The study was arbitrarily divided into consecutive 5-day periods which numbered 1 to 49, inclusive. A basal dietary was arranged for each 5-day term and repeated period after period. The menu for each of the 5 days was different but the food for each 5-day period was identical in kind, amount, and order of use. A few foods of negligible iron content per serving, puffed rice, angel-food cake, white sugar, butter and whipping cream, were permitted ad libitum to meet individual caloric requirements. Since only foods which were low in iron could be used, beef and many fruits and vegetables were excluded. This resulted in a monotonous though not unpalatable diet which required ingenuity to prepare and a scientific viewpoint to consume month after month.

Every effort was made to secure foods of uniform composition. Most of the food was purchased at one time. Individual portions of perishable foods were weighed into wax paper cups and stored in a freezing locker; peaches and tomatoes were canned with distilled water in glass containers; vegetable soup and applesauce [3] were secured in glass containers; the milk [4] and citrus fruit were sampled daily for analysis.

Certain special precautions were taken in order to avoid increasing the iron content of the diet by contamination from the usual sources. All food preparation was done in Pyrex oven or flameware,[5] all china and cooking utensils were rinsed

[3] Given by Beech-Nut Packing Company.
[4] Dairy products were given by Roberts Dairy, Lincoln.
[5] Given by the Corning Glass Works.

in distilled water and the girls used distilled water entirely for food preparation and drinking.

The diet furnished daily: 67 gm. of protein, 1.2 gm. of calcium, 1.5 gm. of phosphorus and 3.5 mg. of iron. A vitamin concentrate [6] containing 6200 U.S.P. units of vitamin A, 900 U.S.P. units of D, 75 International units of B_1, 200 International units of C, and 20 Sherman units of G (riboflavin) was taken daily together with 1 mg. of thiamine chloride.[7] In order to insure that iron would be the only limiting factor in the diet, a supplement of copper sulfate solution to provide 2 mg. of copper daily was also taken.

Changes were made in the basal dietary from time to time in an effort to determine the relation of such a change to the blood picture and to the excretion of iron. During periods 32, 33, 34, 116 gm. of lean beef [8] replaced the 750 ml. of milk daily. This quantity of meat contained the same amount of protein as the milk. During the next three periods, 35, 36 and 37, 232 gm. of meat were used daily while the milk and part of the egg white and cottage cheese were omitted to keep the protein level of the diet constant. Since the iron in the serum and the amount of iron excreted changed when the subjects were on the meat regimen, it was repeated during periods 47, 48 and 49 when 116 gm. of meat were again used daily in place of 750 ml. of milk.

Collections of all stools, urine, and menses were made during the entire study.

The following materials were made into smooth brown digests with 20% hydrochloric acid and then adjusted to a known volume: (1) the composite samples of feces for each 5-day period, (2) the individual samples of foods, (3) the mixtures of the daily samples of milk and citrus fruit taken as representing the 5-day periods, and (4) the menses. A 25 ml. aliquot of the brown digest was pipetted into a 300 ml. Kjeldahl flask and the flask placed in a Pyrex fume duct.

Concentrated nitric acid was used to digest the easily oxidizable material and perchloric acid for that which was difficult to destroy. The resulting salt solution was boiled with water or 2% sodium sulfite to remove any excess perchloric acid. The solution was filtered and made to a final volume of 100 ml. In this solution iron was determined as the thiocyanate by the method developed by Stugart ('31). A Sanford-Sheard Photelometer, which had been calibrated with solutions of known iron concentration, was used for making the color comparisons. The analytical work was done in a laboratory especially designed and equipped to preclude iron contamination during the preparation and analysis of the materials.

Certain blood determinations formed an important part of this study. Hemoglobin content together with red and white cell counts was determined each week. For these 0.5 ml. of venous blood was taken from subjects A, B and D and capillary blood from a finger prick from subject C. About once a month the iron content of the blood serum and the cell volume were determined and smears made for differential leucocyte and reticulocyte counts. At this time 35 to 50 ml. of venous blood were taken from each subject except subject C.

The methods as set forth in "Clinical Diagnosis by Laboratory Methods" by Todd and Sanford ('37) were followed closely for hemoglobin, cell counts, and cell volume. Hemoglobin was converted into oxyhemoglobin in 0.1% sodium carbonate solution and read in the aforementioned Photelometer. The method used for the serum iron determinations was that developed by Moore and Arrowsmith ('37). The blood was collected in paraffined tubes and centrifuged for 45 minutes. Tenth normal HCl was added to ionize the iron and then the protein was precipitated with 20% trichloracetic acid. The deproteinized serum was digested with H_2SO_4 and H_2O_2. In the resulting clear solution, iron was determined as the thiocyanate.

RESULTS

The data for the entire study are summarized in table 1, to show the total intakes and losses of iron for each of the

TABLE 1

Intake, loss and balance and blood data of four subjects at different levels of iron intake

SUB-JECT	DAILY DIET	PERIOD	NO. DAYS	AVERAGE DAILY INTAKE OF IRON (mg.)	TOTAL MG. OF IRON						In venous blood samples	HEMOGLOBIN GM./100 ML.		B. B. C. M/CU.MM.	
					Intake	Fecal excr.	Urinary excr.	Menstrual loss	Total loss	Balance		Begin	End	Begin	End
A	Low-iron	1–19 inc.	94	3.06	287.64	287.64	6.06	29.40	323.10	− 35.46	67.17	15.0	13.7	5.07	4.03
	Low-iron	20–31 inc.	60	3.09	185.40	213.00	4.26	16.40	233.66	− 48.26	95.31	14.3	13.6	4.13	4.43
	Low-iron plus 116 gm. beef	32–34 inc.	15	6.54	98.10	54.45	1.38	4.00[1]	59.83	+ 38.27	23.45	13.4			4.23
	Low-iron plus 232 gm. beef	35–37 inc.	15	9.17	137.55	93.60	2.02	4.00	99.62	+ 37.98		14.2	13.7	4.47	4.57
B	Low-iron	1–19 inc.	94	3.41	320.54	281.06	5.92	46.72	333.70	− 13.16	69.04	15.0	14.5	5.41	4.31
	Low-iron	20–31 inc.	60	3.37	202.20	187.80	3.76	26.70	218.26	− 16.06	94.97	14.3	13.5	4.38	4.34
	Low-iron plus 116 gm. beef	32–34 inc.	15	6.76	101.40	54.45	1.28	5.10[1]	60.83	+ 40.57	22.94	12.9			4.22
	Low-iron plus 232 gm. beef	35–37 inc.	15	9.58	143.85	78.75	1.57	5.10[1]	85.42	+ 58.43			13.4	4.48	4.42
	Low-iron	38–40 / 42–46 inc.	40	4.46	178.40	144.00	3.08	11.60	158.68	+ 19.72	21.94	13.3	13.8		4.26
	Low-iron plus 120 mg. iron as FeSO₄	41	5	124.41	622.05	330.00	0.45	....	330.45	+291.60					
C	Low-iron plus 116 gm. beef	47–49 inc.	15	6.47	97.05	58.50	1.37	7.80[1]	67.67	+ 29.38	22.78	13.5		5.03	4.37
	Low-iron	1–19 inc.	94	3.34	313.96	313.02	6.76	27.08	346.86	− 32.90	40.24	15.0	12.9	4.34	4.01
	Low-iron	33–37 inc.	25	3.96	99.00	95.75	1.91	8.40	106.06	− 7.06		13.2	13.0		4.05
D	Low-iron	35–42 / 45–46 inc.	50	4.45	222.50	187.50	4.00	66.40	257.90	− 35.40	46.57	13.8	12.6	4.66	4.13
	Low-iron plus 116 gm. beef	47–49 inc.	15	6.43	96.45	62.55	1.25	12.20	76.00	+ 20.45	22.28	13.3	12.5	3.98	4.08
All A, B and D	Low-iron		517	3.50		Average daily			3.83	− 0.33					
	Low-iron plus 116 gm. beef daily		60	6.55					4.41	+ 2.14					

[1] One-half of the menstrual loss for the current cycle.

subjects at the different levels of iron intake. The values for the iron content of the urine were calculated from the results of analyses of the composite samples for the first ten periods for each subject.[9] The iron loss from venipuncture was calculated from the amount of blood withdrawn and its hemoglobin content at the time. This loss is not included in the total of usual losses but placed in a separate column beside the figures showing the relation or balance between intake and usual losses. The hemoglobin and red cell values at the beginning and end of each dietary regimen are also given.

Several significant facts are emphasized by the figures in table 1. There were seven different groups of periods when the subjects were on the low-iron diet for periods varying in length from 25 to 94 consecutive days. During this time the intake was never large enough to cover the total of the fecal, urinary and menstrual losses. Considering all the subjects for this time, 517 metabolism days, the intake and total loss calculated on average daily basis, were 3.50 mg. and 3.83 mg. respectively. The negative balance, 0.33 mg. iron daily, would indicate that this amount was being used from the body stores of the metal.

The figures for the daily metabolism of subjects A, B and D when they were eating beef, have been summarized in table 2,

TABLE 2

Average daily iron intake and excretion during 15-day periods of beef intake

SUBJECT	BEEF INTAKE	IRON INTAKE	IRON EXCRETION	IRON RETENTION	
	gm.	mg.	mg.	mg.	%
A	116	6.54	3.63	2.91	44.49
B	116	6.76	3.63	3.13	46.30
	116	6.47	3.90	2.57	39.72
D	116	6.43	4.17	2.26	35.15
Average		6.55	3.83	2.72	41.42
A	232	9.17	6.24	2.93	31.95
B	232	9.58	5.25	4.33	45.25
Average		9.37	5.74	3.63	38.60

[9] The urinary excretion of iron under normal conditions has been found by many workers to be small and constant. This has been so consistently borne out in this laboratory that following analysis on ten urine composites for each subject, iron determinations in urine were discontinued.

which shows the per cent of the iron as well as the number of milligrams which were absorbed during these periods. The average daily intake for the three subjects for the 60 days when 116 gm. of beef were used was 6.55 mg. of iron while the total fecal, urinary and menstrual loss averaged 4.39 mg. of iron. Although figures are insufficient to show whether or not an intake of 9 mg. would promote better absorption than an intake of 6.5 mg. there is no question but that absorption was greater on a daily intake of 6.5 mg. than on intakes ranging from 3.0 to 4.5 mg. The relation of size of intake of iron to the amount absorbed is shown graphically in figure 1.

RELATION OF LEVEL OF INTAKE TO AMOUNT OF IRON RETAINED
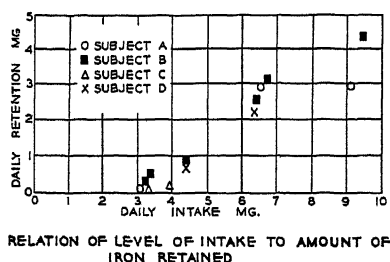
Figure 1

After the first 94 days subject C was not returned to the basal diet but given 120 mg. iron daily as ferrous sulfate in addition to a self-chosen diet for 6 weeks. Her intake, fecal excretion, and retention of iron during this time are shown in table 3. After retaining a total of 2206 mg. during a 6-week period the iron supplement was discontinued and after 5 days she was put on the low-iron diet for 25 days, periods 33–37, inclusive. During this time on an average daily intake of 3.96 mg. she retained 0.13 daily.

TABLE 3

Absorption of iron on a daily supplement of 120 mg. from ferrous sulfate

SUBJECT	MG. IRON ABSORBED PER WEEK						TOTAL	PER CENT OF INTAKE [1]
	1	2	3	4	5	6		
							mg.	
C	533	487	606	192	94	294	2206	40.4
D	615	334	226	362	394	222	2153	39.4

[1] Daily diet was assumed to contain 10 mg. iron.

The treatment of subject D was similar to that just described for subject C. At the end of 6 weeks of iron therapy she had retained a total of 2153 mg. of iron (table 3), and on the low-iron diet she retained 0.70 mg. daily.

When subjects B, C and D ate self-chosen diets after the metabolism study, the feces were kept for analysis for 1 or 2 weeks. Subject B had an average daily fecal excretion of 7.70 mg. of iron the first week and 9.37 mg. the second week; subject C excreted 12.05 mg. of iron daily the first week and 10.69 mg. the second; and subject D excreted 9.52 mg. daily the first week.

Throughout the entire study the results of the white cell, differential leucocyte and reticulocyte counts and the determination of cell volume remained within the limits of normal variations, and therefore reflected no measurable effects of either the low iron diet or its supplements.

Probably the most significant results of the study are the values obtained for the iron content of the blood serum of the subjects at intervals during the 8 months. These are charted in figure 2. They show a gradual and consistent drop

SERUM IRON VALUES FOR THREE SUBJECTS
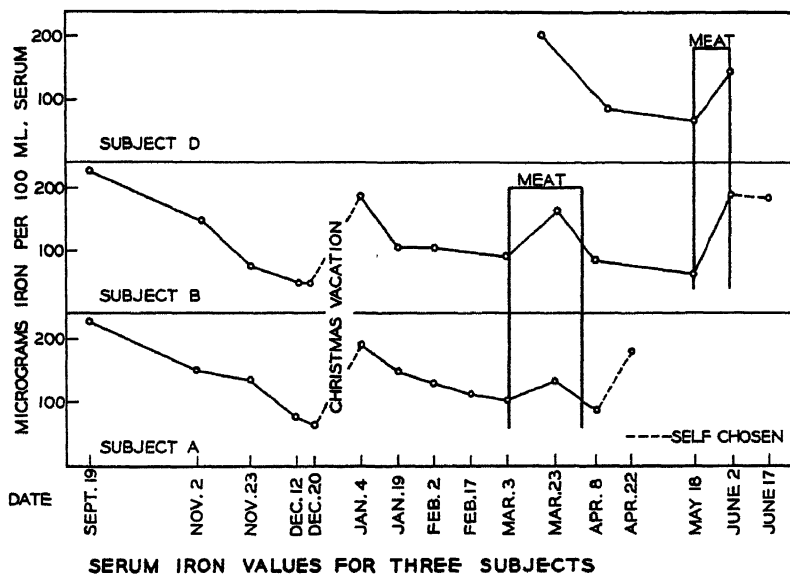
Figure 2

in the serum iron during the low-iron regimen and a rise when the diet was self-chosen and when the iron content of the diet was increased by the use of beef.

At the beginning of the study the serum iron value for subject A was 227 µg. per cent and decreased to 65 µg. per cent at the end of 92 days. Subject B started with a value of 228 µg. per cent and this dropped to 50 µg. per cent in the 92 days. During 13 days of vacation, on a self-chosen diet, these values rose sharply to 190 and 189 µg. per cent for subject A and B respectively. Following vacation they again decreased and continued to decrease as the time on the low-iron diet increased. Their response was very similar to that in the fall when the study was begun. Thus, in the fall after 65 days on the low-iron diet the serum iron value of subject A had dropped 89 µg. per cent and it dropped 88 µg. per cent in the 59 days from January to March. Similarly, for subject B there had been a decrease of 92 µg. the first 59 days following Christmas vacation. Because of the difficulty of a venipuncture serum iron determinations were not made on subject C except at the end of the 94 days on the low-iron diet. At that time it was 81 µg. per cent. The serum value of subject D on a self-chosen diet and after she had taken 120 mg. iron daily for 6 weeks was 211 µg. per cent. After 25 days on the low-iron diet it had dropped to 88 µg. per cent and after 61 days to 68 µg. per cent.

The effect of adding beef as a source of iron in the diet was reflected similarly in the iron content of the blood serum of all three subjects, A, B and D. The serum iron value of subject A was determined after 59 days on the low-iron diet and was found to be 102 µg. per cent. The next 19 days she was given beef, 116 gm. daily for 15 days and 232 gm. for 4 days. At the end of this time her serum level had risen to 135 µg. per cent of iron. After 5 days on the low-iron diet her serum iron value had decreased to 87 µg. per cent, but after the next 14 days while she was on a self-chosen diet, it rose to 180 µg. per cent. The serum level of subject B before the meat was given was 97 µg. of iron and after 19 days on the

meat, 116 gm. daily for 15 days and 232 gm. for 4 days, it had risen to 162 µg. per cent. After 5 days on the low-iron diet her serum iron was 84 µg. per cent and at the end of 45 days it was 65 µg. per cent. At this point she was again given 116 gm. of beef daily for 15 days which increased the serum iron to 190 µg. per cent. This was followed by 15 days on a self-chosen diet which gave a value of 188 µg. per cent. After 2 months on a self-chosen diet her serum iron level was 220 µg. per cent which was comparable to the 228 µg. per cent it had been 8 months earlier when the study was begun. The serum iron value for subject D was 68 µg. per cent after 61 days on the low-iron diet and after 15 days of eating 116 gm. beef daily this value had risen to 144 µg. per cent. It was not possible to make determinations later on a self-chosen diet.

DISCUSSION

Discussion of the results will be limited to the relation of intake to excretion of iron, the significance of blood serum iron values on different levels of iron intakes, and the application of these to the interpretation of iron requirement.

Considering the entire study, with the omission of subject A, it appears that the intestine normally does not excrete iron and it follows that the fecal excretion of this metal would not exceed the intake over any period of observation long enough to counteract variations due to intestinal motility. If this is true, the size of fecal excretion cannot be used as a criterion on which to base dietary recommendations. Although the fecal excretions on the intake of approximately 6.5 mg. of iron were almost the same as those on 3.5 mg. this relationship does not continue as the intake increases to 9 mg. for here the fecal excretion increases to 5 and 6 mg. and on self-chosen diets to anywhere from 7 to 12 mg.

Certain additions to the basal diet may explain the negative iron balance of subject A during periods 20–31 inclusive when her daily intake and excretion averaged 3.09 mg. and 3.55 mg. respectively. During this time subject A digressed slightly from the basal diet. Since her fecal excretions never exceeded

4.9 mg. of iron daily and her serum iron values continued to decrease, the digressions were not considered of sufficient magnitude to require the omission of the metabolism figures from this report. They are not included, however, in the generalizations.

A marked decrease in hemoglobin or red cell content was not to be expected on the low-iron dietary since it has been shown that in case of a dietary restriction of iron, the depots of iron are probably used before hemoglobin production suffers. The slow response or resistance to change of hemoglobin level during the study may have been a reflection of the optimal protein and vitamin intake, or more especially the copper supplement.

Though the decrease in serum iron from the time of one determination to the next for the subject on this study did not always exceed the normal variation of 41 μg. per cent reported by Moore, Minnich and Welch ('39) the total change was always greater. For instance, the response of subjects A, B and D when changing from a self-chosen diet to the low-iron regimen involved decreases of 162, 178, and 143 μg. per cent respectively in 92 days for A and B and 61 days for D.

The decrease in serum iron values was not paralleled with a decrease in the rate of absorption of iron for as the study progressed the iron absorbed from the constant low intake did not decrease. This continued decrease in serum values is not exactly in keeping with the concept of Moore, Minnich and Welch ('39) that a state of equilibrium exists between the iron being absorbed, the adequacy of the iron stores, and the rate of hemoglobin synthesis. The total metabolism figures showed a negative iron balance on the low intakes, and a slight consistent decrease in hemoglobin values accompanied the low serum iron concentrations but iron absorption was not correspondingly affected. Apparently the iron in the serum is more labile than that which is stored and when iron demands are not met with corresponding intakes, the drain on the serum iron is greater than its replenishment from the storage depots.

The present concept of serum iron together with the results of this study suggests the possibility of conducting iron metabolism studies by determining serum iron values during different levels of iron intake. If the iron deficit or balance between intake and total loss is indicated by the iron content of the serum there would be no need for collection and analysis of feces, urine, and menstrual blood.

These data may contribute to the understanding of the iron requirement of normal women. When all the periods on the low-iron diet for all the subjects are considered, the average daily intake was 3.50 mg. of iron and the average daily total of fecal, urinary and menstrual loss was 3.83 mg. This indicates that a daily intake of 3.50 mg. of iron was not sufficient to cover the losses and that iron was being drawn from body tissues or blood. This is further substantiated by the fact that on this intake the blood serum iron consistently decreased from the upper to the lower limits of normality, and that the hemoglobin and red cell values also decreased, consistently though not strikingly.

From a practical standpoint a daily intake of 3.5 mg. of iron is seldom encountered. Even the most haphazardly chosen diet, unless made up almost solely of refined cereals, will contain more than this amount of iron. It is of scientific importance, however, to realize that an optimal intake of all known dietary essentials except iron can fail to meet the requirements of the female body. In other words, there still exists a need for iron which cannot be met by other dietary constituents.

Increasing the daily intake of iron of three of the subjects to 6.55 mg. by the use of 116 gm. of beef completely changed their metabolic picture and this level of intake might be considered adequate for these subjects. The average daily total loss of 4.41 mg. was only slightly more than the 3.83 mg. on the lower intake, an average daily retention of 2.14 mg. of iron occurred, and the serum iron values rose significantly.

Although the results may not definitely establish the adequacy of 6.5 mg. of iron daily for young women in general,

the finding that 6.5 mg. provided 2 mg. daily in excess of the total loss of these subjects indicates that this level of intake would still allow for individual variations in amount of menstrual loss, rate of intestinal motility, variations in food composition and losses in cooking.

The results of the entire study suggest that there is greater need for emphasis on a diet liberal in other minerals, protein, and vitamins than on iron as a single item. Contrary to previous interpretations of iron metabolism data, it appears that the amount of iron may not warrant special consideration in dietary practices or recommendations but that an ordinary mixed diet adequate in the other known essentials would supply ample iron for maintenance and replacement in the normal young woman.

SUMMARY AND CONCLUSIONS

The iron metabolism of four healthy college girls was studied for periods varying from 3 to 8 months. The greater part of the time the subjects were on a diet adequate in all known dietary essentials except that it contained only 3.5 mg. to 4.5 mg. of iron.

A fecal excretion of iron which did not exceed the iron intake did not mean the body was in iron equilibrium. At all times on the low-iron diet the total of fecal, urinary and menstrual iron losses exceeded the intake so that the body was losing iron from its storage depots. The extent of this loss averaged 0.33 mg. of iron daily on an intake of 3.50 mg.

When the iron content of the diet was increased to 6.55 mg. by replacing the 750 ml. of milk in the basal diet of three subjects with 116 gm. of lean beef, there was an average daily retention of 2.14 mg. of iron.

The iron content of the blood serum decreased consistently and significantly during the low-iron regimen but whenever the intake of iron was increased the serum iron levels rose markedly and then dropped again when the low iron intake was resumed.

The results suggest that there is no need for emphasis on amounts of iron exceeding 6.5 mg. daily in the diet of normal young women; emphasis should rather be placed on liberal amounts of other dietary essentials.

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CHOLINE METABOLISM

VII. SOME DIETARY FACTORS AFFECTING THE INCIDENCE AND SEVERITY OF HEMORRHAGIC DEGENERATION IN YOUNG RATS

WENDELL H. GRIFFITH AND DWIGHT J. MULFORD

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri

ONE FIGURE

A dietary deficiency of choline and of the "labile methyl supply" produces in young rats a severe pathological state which has been named hemorrhagic degeneration. The prevention of the characteristic renal lesions and of the deposition of excess liver fat is a satisfactory test for choline and other sources of labile methyl, such as, methionine and betaine. Previous papers have considered the effect in these experiments of the age and sex of rats (Griffith, '40 a) and of supplements of cystine (Griffith and Wade, '40) and of cholesterol (Griffith, '40 b). The present report is concerned with the influence of the deficiency of labile methyl on the rate of growth and on the recovery process in surviving animals and with the relation between the food intake and the occurrence of the degenerative condition. In addition, the effect of certain dietary factors has been investigated, preparatory to using the production and prevention of hemorrhagic degeneration in an extensive study of the relation of the labile methyl supply to the metabolism of choline.

EXPERIMENTAL

The experimental procedures were similar to those previously described (Griffith and Wade, '39). The appearance of the kidneys was noted at the end of the experimental period

and the per cent of animals in each group showing the hemorrhagic state (Griffith, '40 a) is recorded in tables 2 to 5 under the heading "renal lesions". Determinations were made of liver and kidney weights and of the total chloroform-soluble substances in the liver. The term liver fat in tables 3 and 5 refers to this fraction. The values shown in figure 1 for nonprotein nitrogen (Koch and McMeekin, '24), amino-acid nitrogen (Folin, '22) and glucose (Shaffer and Somogyi, '33) were obtained by the analysis of tungstic acid filtrates of pooled blood from groups of four or more rats.

Young male rats, 20 days of age and 25 to 34 gm. in weight were used in the experiments recorded in table 4. Rats, 21 to 26 days of age and 38 to 42 gm. in weight, were used in the experiments recorded in figure 1 and in tables 2, 3 and 5. The basal diets are described in table 1. The stock diet consisted of a commercial dog food.[1]

TABLE 1

Composition of diets

	AA[1]	AO 39 [1]	AC 32	AC 50	AC 51	CG 3
	%	%	%	%	%	%
Amino acid mixture [2]	24	0	0	0	0	0
Purified casein	0	18	15	18	18	15
Gelatin	0	0	0	0	0	3
Cystine	0	0	0.3	0	0.3	0.1
Cholesterol	0	0	0	0	0	0.5
Lard	20	20	9	20	20	20
Sucrose (commercial)	48.9	54.9	62.6	48.9	48.6	49.3
Dried brewer's yeast	0	0	6	6	6	6
Salt mixture [3]	4	4	4	4	4	4
Calcium carbonate	1	1	1	1	1	0
Agar	2	2	2	2	2	2
Natola [4]	0.1	0.1	0.1	0.1	0.1	0.1

[1] Water soluble vitamins supplied by daily oral administration of 0.02 mg. of thiamine chloride, 0.02 mg. of riboflavin, 0.02 mg. of pyridoxine and 0.5 mg. of nicotinic acid.

[2] Prepared according to McCoy and Rose ('37) except that methionine and cystine were omitted.

[3] Hawk and Oser ('31).

[4] Fortified fish liver oil.

[1] Purina dog chow.

RESULTS

Effect of hemorrhagic degeneration on rate of growth.
Young rats fed low choline and low labile methyl food mixtures
develop fatty livers within 48 hours and hemorrhagic lesions
within 3 to 6 days. The renal pathology is most severe between
the sixth and eighth days and either results in the death of the
animal or in a spontaneous recovery from the hemorrhagic
state. During this critical stage the animal is noticeably sick
and there is a spectacular rise in the nonprotein nitrogen of
the blood. Figure 1 shows the daily change in weight of rats
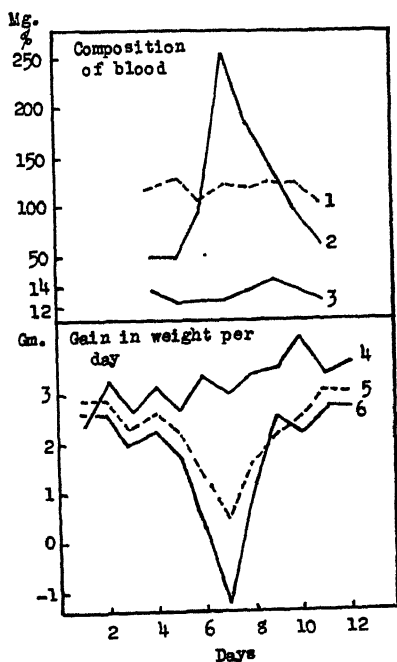
Fig. 1 The effect of hemorrhagic degeneration on the composition of blood
and on the rate of growth of 40-gm. male rats. The analysis of blood, collected
after decapitation, was made on pooled samples from individual groups of four
or more rats maintained on diet AC 32 for 4 to 11 days. The daily changes in body
weight were determined in other groups continued on the diet for 12 days.
    Curve 1. Glucose of blood.
    Curve 2. Nonprotein nitrogen of blood.
    Curve 3. Amino-acid nitrogen of blood.
    Curve 4. Average daily gain in weight of a control group of thirteen rats fed
diet AC 32 plus 0.3% choline chloride.
    Curve 5. Average daily gain in weight of a group of twenty-two rats fed
diet AC 32.
    Curve 6. Average daily gain in weight of twelve of the more severely affected
rats in the group represented in curve 5.

fed diet AC 32, with and without added choline, and also the levels of blood glucose, amino acid nitrogen and nonprotein nitrogen in similar groups fed diet AC 32 for 4 to 11 days. The rapid decrease in the nonprotein nitrogen and the restoration of growth are noteworthy features of the recovery phase. Similar observations were made by Cox, Smythe and Fishback ('29) in an investigation of the toxic effect in rats of a dietary supplement of cystine.

Rats which survived the acute stage were continued on experimental diets in order to determine the extent of the recovery process. Table 2 shows results obtained with diets

TABLE 2

The effect of hemorrhagic degeneration on rate of growth of 40-gm. male rats

GROUP	DIET	EXPERI-MENTAL PERIOD	NO. OF RATS [1]	FINAL WEIGHT	WEIGHT AS PER CENT OF BODY WEIGHT		RENAL LESIONS
					Kidneys	Liver	
		days		*gm.*			*%*
1	AC 32	7	23–21	56	1.55	7.05	96
2	AC 39	7	18–16	50	1.77	6.82	100
3	AC 32	10	27–21	60	1.80	7.45	100
4	AC 39	10	26–17	45	2.28	7.67	100
5	AC 39, 7 days + AC 32, 3 days	10	32–17	50	2.12	7.54	100
6	AC 39 + 0.3% choline chloride	10	15–15	61	1.01	4.38	0
7	AC 32	30	34–26	145	0.96	8.80	50
8	AC 39, 7 days + AC 32, 23 days	30	25–11	131	0.97	9.80	100
9	AC 39, 7 days + AC 32, 3 days + Stock diet, 20 days	30	43–21	134	1.15	5.24	88
10	AC 32 + 0.3% choline chloride	30	10–10	153	0.88	4.80	0
11	Stock diet	30	19–19	140	0.91	4.30	0

[1] Second figure shows the number of surviving rats.

AC 32 and AC 39. The latter was used in order to produce greater damage to the kidneys than was possible with AC 32. Groups 1 to 5, table 2, show the 7- and 10-day effects of the two diets, effects which are prevented by choline (group 6). The average daily food intakes during the first and second 5-day periods were 5.4 and 3.3 gm. for group 3 (diet AC 32) and 4.5 and 1.4 gm. for group 4 (diet AC 39). This decrease in food consumption during the second half of the experi-

mental period was prevented by choline (group 6), the values for the two 5-day periods being 4.5 and 5.4 gm. for this group. The more severe effect obtained with diet AC 39 is believed to be due to the omission of supplies of labile methyl occurring in yeast rather than to the fact that the vitamin supplement lacked members of the B complex. In these experiments the increased weight of the liver is used as an index of the deposition of excess fat. Comparison of groups 7 to 11, table 2, demonstrates that the rats which survived hemorrhagic degeneration during the 10-day period grew nearly as well on the injurious AC 32 ration during a subsequent 20-day period as normal rats on the stock diet (group 11). The liver weight remained excessive in groups 8 and 9 but decreased in group 10 which was not continued on the low choline regime. The marked increase in kidney weight occurring during the hemorrhagic phase was no longer evident in the 30-day animals. Although all rats on diet AC 32 showed renal hemorrhage after 10 days, in 50% of a similar group the kidneys appeared normal after 30 days (groups 3 and 7). Such extensive renal repair did not occur in rats suffering the more severe effect of diet AC 39 in the first 10 days (groups 8 and 9). The kidneys of most of these animals showed the characteristic discoloration, mottling or scarring (without hemorrhage). Similar unpublished results have been obtained in rats fed the stock diet for 15 months following the preliminary 10-day period on diet AC 39.

Although the experiments in table 2 indicate that rats which survive acute renal degeneration may continue to grow in spite of the persisting renal damage, the continuation of growth depends upon the diet and upon the severity of the original renal lesions. This is illustrated by the results obtained following the inclusion of cholesterol in diets AC 51 and CG 3 (table 3). Groups 1 to 5 show the opposing effects of choline and cholesterol and the protective action of choline in diet AC 51. The average daily food intakes of the rats in groups 1 to 5 were 4.2, 5.1, 5.3, 5.5 and 5.4 gm. respectively. Groups 6 to 16 were fed CG 3, the most severe diet which has

been used in this study. The average daily food intakes of groups 6 to 9 were 4.9, 5.1, 5.1 and 3.8 gm. respectively. It is of significance that the food consumption was not affected until after the appearance of the renal lesions. The liver fat was excessive on the second day, renal hemorrhage occurred after 3 days and 25% of the animals were dead by the eighth day (groups 6 to 9). These remarkable effects, which were prevented by choline (groups 10 and 11), were intensified by the presence of cholesterol in a diet in which the labile methyl supply had been diminished by decreasing the level of the high-methionine protein, casein. The damaging effects of this diet continued to appear during the so-called recovery phase. Only nine rats out of twenty-seven (group 16) survived for 75 days, the highest mortality occurring between the sixth and ninth and between the thirty-fifth and forty-fifth days. In the nine surviving animals, the livers were exceptionally fatty and the kidneys were grossly and microscopically abnormal. One rat showed evidence of recent renal hemorrhage.

The results with diet CG 3 were also unusual because for the first time in our experiments, choline failed to afford complete protection. This failure was not at all evident during the crucial 8-day period but appeared during a subsequent 22-day period. Groups 12 to 15, table 3, showed an increasing level of chloroform soluble substances in the liver and in four of the eleven rats in group 15 renal hemorrhage, as well as ocular hemorrhage, occurred. These later effects appearing in rats on diets containing cholesterol and choline are being investigated further.

Effect of food consumption on hemorrhagic degeneration. Table 4 (groups 1 to 3) shows the effect of a diet (AA) in which protein was replaced by an amino-acid mixture lacking the sulphur-containing acids. The addition of cystine increased the incidence of renal lesions and these were prevented by methionine. The moderate injury occurring in group 1 seemed unusual in view of the fact that this food mixture was very deficient in supplies of labile methyl. Diet AA was an unsatisfactory ration, possibly because of impalatability and

TABLE 3

The effect of cholesterol on severity of hemorrhagic degeneration in 40-gm. male rats

GROUP [1]	EXPERI- MENTAL PERIOD	CHOLINE CHLORIDE ADDED PER GRAM OF FOOD	NO. OF RATS [2]	FINAL BODY WEIGHT	KIDNEY WEIGHT AS PER CENT OF BODY WEIGHT	LIVER FAT AS PER CENT OF LIVER	RENAL LESIONS
	days	mg.		gm.			%
1	8	0	21–18	51	2.10	21.5	95
2	8	0.35	21–20	63	1.42	20.5	62
3	8	0.5	21–21	66	1.18	17.7	38
4 [3]	8	0.5	21–21	69	1.05	13.0	0
5	8	1.0	21–21	66	1.08	8.8	0
6	2	0	16–16	42	1.29	16.8	0
7	3	0	15–15	47	1.27	18.4	7
8	4	0	14–14	49	1.36	26.7	71
9	8	0	24–18	47	2.38	26.9	100
10	8	4	21–21	62	1.14	5.5	0
11	8	6	14–14	65	1.04	4.9	0
12	15	4	18–18	97	0.98	6.1	0
13	25	4	22–22	116	1.03	8.6	0
14	25	6	24–24	122	0.99	8.6	0
15	40	4	11–11	176	0.86	9.8	36
16	75	0	27– 9	190	0.92	34.9	100

[1] Groups 1 to 5, Basal AC 51 + 1% cholesterol. Groups 6 to 16, Basal CG 3 (0.5% cholesterol).

[2] Second figure shows the number of surviving rats.

[3] Cholesterol omitted.

lack of sulphur amino-acids, and the rats in group 1 consumed too little food for even moderate growth. This failure of appetite was evident from the first day of the experimental period. The average food intakes for the first 4 and the last 3 days were 2.1 and 2.0 gm. respectively.. The corresponding food intakes of group 4 (diet AC 39) were 3.6 and 1.9 gm. This diet which differed from AA in the use of casein instead of the amino-acid mixture was consumed in larger amounts at the start of the experiment so that there was some increase in body weight even though the later food intake was no more than that found in group 1. These results suggested the possibility that a deficiency of choline or of labile methyl would result in hemorrhagic degeneration only if the metabolic demands of the maintenance and growth processes were great enough to require more labile methyl than was supplied in

TABLE 4

The effect of food intake and of various antihemorrhagic factors on occurrence of hemorrhagic degeneration in 20-day-old male rats during. a 7-day experimental period

GROUP	DIET	NO. OF RATS	AVERAGE BODY WEIGHT Initial	AVERAGE BODY WEIGHT Final	AVERAGE FOOD PER DAY	WEIGHT AS PER CENT OF BODY WEIGHT Kidneys	WEIGHT AS PER CENT OF BODY WEIGHT Liver	RENAL LESION:
			gm.	gm.	gm.			%
1	AA	8	30	29	2.1	1.40	5.5	13
2	AA + 0.3% cystine	16	30	30	2.0	1.72	5.4	69
3	AA + 0.3% cystine and							
	0.6% dl-methionine	20	29	32	2.3	1.46	4.4	0
4	AC 39	10	30	36	2.9	2.37	7.6	100
5	AC 39 + 6% yeast [1]	10	30	47	3.9	1.47	6.4	100
6	AC 39 +							
	0.3% choline chloride	10	30	44	3.7	1.18	4.1	0
7	AC 39. Food restricted	10	31	34	2.4	1.77	6.8	100
8	AC 39. Food restricted	10	31	32	1.9	1.17	4.6	10
9	AC 32	19	29	40	3.3	1.90	6.8	90
10	AC 32. Food restricted	20	30	35	2.0	1.23	4.9	0
11	AC 32 + vitamin K [2]	12	27	39	..	1.89	8.3	92
12	AC 32 + vitamin K [3]	10	27	38	..	2.04	7.0	90
13	AC 32 + ascorbic acid [4]	21	29	41	3.8	2.0	7.4	93
14	AC 32 + vitamin P [5]	14	28	41	3.9	1.79	7.5	93
15	AC 32 + vitamin P [6]	7	30	45	3.6	1.70	7.3	100
16	AC 32 + 0.038%							
	choline chloride	22	30	52	5.3	1.20	6.1	0
17	Stock diet, 8-day period	21	40	68	..	1.21	4.6	0
18	Stock diet, 5.5 days.							
	Fasting, 2.5 days	21	40	42	..	1.37	4.0	0

[1] 6% yeast in place of vitamin supplement.

[2] Oral administration of 100 chick units of vitamin K_1 per day in form of an alfalfa extract (oil).

[3] Subcutaneous injection of 400 chick units of the potent oil per day for first 4 days. Daily intraperitoneal injection of 0.3 mg. of 4-amino-2 methyl-l-naphthol hydrochloride.

[4] Oral administration of 1 mg. of ascorbic acid per day. Also 1 mg. added per gram of food.

[5] 5 mg. of "hesperidin" per gram of food.

[6] 20 mg. of "lemon citrin" per gram of food.

the ration. The definite relation between the occurrence of renal lesions, the level of ingested food and the rate of growth was demonstrated by the absence of renal pathology in rats fed restricted amounts of diets which were highly injurious if fed ad libitum. Renal lesions were severe and the liver weight was markedly increased on diet AC 39 (group 4) even if the incomplete vitamin supplement was replaced by yeast (group 5). These effects also occurred if the food intake was restricted to 2.4 gm. per day but not if the food was limited to 1.9 gm. per day (groups 7 and 8). Similarly, restriction of group 10 to 2.0 gm. of diet AC 32 per day resulted in complete protection whereas the unlimited consumption of the same diet produced the characteristic renal damage and increase in liver weight (group 9). Fasting for 60 hours was without evident effect upon liver and kidney weights, expressed as per cent of body weight (groups 17 and 18).

Effect of antihemorrhagic dietary factors on hemorrhagic degeneration. The occurrence of renal and ocular hemorrhage in rats on a low choline, low labile methyl diet made it desirable to determine the possible effect of other antihemorrhagic factors. The results in table 4, groups 11 to 15, indicated that hemorrhagic degeneration was unaffected by vitamin K, by ascorbic acid and by material supplying a possible "vitamin P".

Effect of thiamine chloride, riboflavin, pyridoxine, calcium pantothenate and nicotinic acid on hemorrhagic degeneration. The influence of these members of the vitamin B complex was determined by noting the effect of supplements of each in diet AC 50, alone and with partially protective levels of choline (table 5). Basal diet AC 50 contained 6% of yeast so that in these experiments the individual vitamins were added to a diet already adequate, except in choline. The ingestion of extra thiamine, riboflavin, pyridoxine and calcium pantothenate was without significant effect upon the severity of the choline deficiency. However, the two levels of choline used in these experiments were not as protective in the diets containing nicotinic acid as in the control diets. The sensitivity of

TABLE 5

The effect of vitamins of the B-complex on severity of hemorrhagic degeneration in male rats, 40 gm. in weight and 21 to 26 days of age, during an 8-day experimental period

DIET [1]	NO. OF RATS	FINAL BODY WEIGHT	AVERAGE FOOD INTAKE PER DAY	KIDNEY WEIGHT AS PER CENT OF BODY WEIGHT	LIVER FAT AS PER CENT OF LIVER	RENAL LESIONS
		gm.	gm.			%
Basal	42	50	4.3	2.01	18.0	93
+ Thiamine chloride	42	53	4.4	1.91	21.6	86
+ Riboflavin	43	53	4.5	1.97	20.5	98
+ Pyridoxine	21	55	4.6	1.65	21.2	81
+ Calcium pantothenate	42	51	4.3	2.02	21.1	98
+ Nicotinic acid	43	49	4.4	2.20	22.3	95
Basal with 0.02% choline chloride	42	59	5.0	1.28	19.9	52
+ Thiamine chloride	42	62	5.1	1.28	19.7	69
+ Riboflavin	42	60	5.1	1.41	19.8	64
+ Pyridoxine	42	61	5.2	1.39	19.0	79
+ Calcium pantothenate	42	60	5.0	1.29	16.9	62
+ Nicotinic acid	21	56	4.8	1.61	19.9	90 .
Basal with 0.04% choline chloride	21	63	5.3	1.09	12.5	10
+ Thiamine chloride	21	65	5.4	1.03	12.5	10
+ Riboflavin	21	66	5.5	1.00	12.8	5
+ Pyridoxine	21	67	5.7	0.96	12.6	0
+ Nicotinic acid	21	60	5.2	1.05	19.9	14

[1] Basal diet, AC 50. Indicated vitamin supplements were at the following levels: nicotinic acid, 0.5 mg. per gram of food; all others, 0.05 mg. per gram of food.

these animals to variations in the labile methyl supply suggests a diversion of a part of this supply to the formation of trigonelline as an explanation of the apparent choline-opposing action of nicotinic acid (Ackermann, '12).

DISCUSSION

Hemorrhagic degeneration occurs on certain low choline, low labile methyl diets fed ad libitum but is prevented if the food intake is restricted. This observation is of considerable importance because the low food intake of rats on diets with multiple deficiencies may mask the labile methyl deficiency. Improvement of food consumption, even though slight and

temporary, would stimulate metabolism and thus increase the effect of the methyl deficiency so that hemorrhagic degeneration might occur. Such an effect would not necessarily indicate a direct antagonistic relation between choline or the labile methyl supply and the agent causing the increase in the food intake.

This demonstration of the influence of the food intake and of growth on the effects of chöline deficiency confirms the earlier statements that ''the deposition of liver fat was intensified on those diets permitting the better rates of growth'' (Griffith and Wade, '39), and that ''the renal lesions were produced more readily if the dietary protein was adequate in amount and in composition for good growth'' (Griffith and Wade, '40). In view of our failure to demonstrate an antagonistic relation between thiamine or pyridoxine and choline as reported by McHenry ('37) and by Gyorgy and Goldblatt ('40), respectively, it is suggested that their results may be explained on the basis of an indirect effect of these two vitamins on the food intake or on the rate of growth of the experimental rats. In one experiment, McHenry equalized the food consumption of two groups of rats on a low choline basal diet, with and without supplementary thiamine, and found a greater deposition of liver fat in the thiamine-fed group. These animals, however, grew at a faster rate than the controls fed the same amount of the basal diet and may have used more of the labile methyl supply for the synthesis of methylated compounds other than choline.

The experiments of Griffith and Wade ('39 and '40) demonstrated the importance of choline and of methionine in the maintenance of the normal renal structure in young rats. At the same time, du Vigneaud showed that the methyls of choline (du Vigneaud, Chandler, Moyer and Keppel, '39) and of betaine (Chandler and du Vigneaud, '40) may be transferred to homocysteine and suggested that ''the presence of methyl groups in a utilizable form, such as, methionine or choline, may be essential in the diet'' (du Vigneaud, Chandler, Cohn and Brown, '40). Our own observations support such a con-

clusion and, in addition, point to hemorrhagic degeneration as the specific effect of a dietary deficiency of the labile methyl supply. The various manifestations of hemorrhagic degeneration may be due to the failure of synthesis in the body of one or more of the many methylated compounds important as constituents of tissues, as metabolites or as detoxication products of toxic substances.

Choline is of prime importance as a lipotropic substance and as a source of labile methyl. In this connection it may be of unusual significance that dietary supplements of cholesterol produce injurious effects which appear after 30 to 40 days even though the diet contains more than the amount of choline required for complete protection of the rat during the crucial 8-day period. Possibly, the continued prevention of the toxic effect of cholesterol is dependent upon the presence in the food mixture of choline and of some other indispensable compound, the lack of which becomes evident only in longer experimental periods.

SUMMARY

1. A dietary deficiency of choline and of the labile methyl supply in young rats produces a marked elevation of the nonprotein nitrogen of the blood coincident with renal hemorrhagic degeneration.

2. Survival, renal repair and resumption of growth depend upon the severity of the acute phase.

3. The aggravating effect of dietary cholesterol is prevented by choline during the crucial 8-day period but not during a subsequent 30-day period.

4. Supplements of thiamine, riboflavin, pyridoxine, calcium pantothenate, ascorbic acid, vitamin K and "vitamin P" do not affect the severity of the results of labile methyl deficiency whereas nicotinic acid exerts a moderate choline-opposing action.

5. Hemorrhagic degeneration, which is severe if diets are fed ad libitum, may be prevented by partial restriction of the consumption of food.

6. The deposition of liver fat or the appearance of renal hemorrhage in experiments in which a dietary supplement increases the consumption of food or the rate of growth is not necessarily evidence of a direct antagonism between choline and the dietary supplement.

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SUPPLEMENT

PROCEEDINGS OF THE EIGHTH ANNUAL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

The eighth annual meeting of the American Institute of Nutrition was held in Chicago at the Stevens Hotel on April 16, 1941.

COUNCIL MEETING

The Council meeting was held at the Stevens Hotel, Tuesday afternoon and evening, April 15th, at 4:15 P.M. All members were present. Formal actions of the Council are reported in the minutes of the Business Session.

SCIENTIFIC SESSIONS

The scientific program began at 9:00 A.M. and proceeded on schedule time. All the papers listed on the program were given. President Carpenter presided at the morning session and Vice-President Hogan at the afternoon session. A special feature of the morning session was an address on the topic "Nutrition and Defense" by M. L. Wilson, Chairman of the Advisory Committee to the Coordinator of Health and Nutrition in Relation to Defense Activities.

BUSINESS SESSION

The business meeting was called to order by President Carpenter at 11:30 A.M., April 16th. The reading of the minutes of the previous annual meeting was dispensed with since they had been published in the Journal.

The Secretary announced that the Committee of Judges for the 1940 Mead Johnson Award consisted of Drs. G. O. Burr, C. A. Elvehjem, H. Deuel, R. C. Lewis and W. C. Rose, Chairman, and that its report which had been approved by the Council would be made at the time scheduled in the program.

The report of the Treasurer was read by Dr. W. H. Sebrell. The auditors, Drs. A. H. Smith and Helen S. Mitchell, previously appointed by the President, reported that they had examined the Treasurer's books and found them correct. It was moved and carried that the report of the Treasurer be accepted.

In the absence of the Editor, the Secretary read the report submitted by Doctor Cowgill and called specific attention to his question as to whether the Journal should adopt a more liberal attitude toward food advertising in the interest of additional income. The Secretary reported that the Council expressed doubt regarding the desirability of such a policy. There was no expression of opinion from the meeting.

The Secretary reported that as a result of a mail vote by the Council, the President had appointed Drs. L. A. Maynard, Helen S. Mitchell and Lydia J. Roberts to serve as a Liaison Committee between the recently created Food and Nutrition Committee of the National Research Council and the Council of the Institute, the members of this Liaison Committee being also members of the Food and Nutrition Committee, and that it was voted to continue the Liaison Committee. The Secretary outlined some of the activities of the Food and Nutrition Committee.

The Secretary reported that the Council had been requested to appoint a committee to give technical advice regarding food relief in Europe and that the Council recommended the authorization of the President to appoint a committee to give this advice, when requested, to private agencies engaged in food relief activities in Europe. This recommendation was adopted. President Carpenter appointed the following committee: G. R. Cowgill, Hazel Stiebeling and E. V. McCollum, Chairman.

The By-laws Committee, appointed at the previous meeting of the Institute and consisting of Drs. Grace MacLeod, L. A. Maynard and A. H. Smith, Chairman, reported on changes suggested in the interest of business expediency and better definition of tenure of office, which changes were approved by

the Council and are to be submitted to the members of the Institute for vote before the next meeting. The Secretary also reported that the Council proposed a change in the By-laws increasing the membership limit to 300. The recommendation that these various proposed changes in the By-laws be submitted to the membership by mail vote was adopted.

In accordance with the recommendation of the Council, it was voted that the Institute should be represented in the Division of Biology and Agriculture of the National Research Council, and the President was authorized to appoint a representative for 3 years. President Carpenter appointed Dr. L. A. Maynard as representative.

The Secretary reported the discussion by the Council of Bill H.R. 3871, which would prevent experiments on animals in the District of Columbia. Upon recommendation of the Council, the following resolution was passed: ''It is hereby resolved by the American Institute of Nutrition at its annual meeting in Chicago on April 16, 1941, that the Institute of Nutrition is opposed to the passage of this bill and earnestly urges that it be rejected. The President of the American Institute of Nutrition is directed to inform the Chairman of the Committee for the District of Columbia of the House of Representatives of this action.''

The Secretary announced the following actions by the Council:

1. The appropriation of 5 cents per member, to be taken from the Institute treasury, to defray expenses of the Federation Committee for the Protection of Biological Research, which deals with such matters as Bill H.R. 3871 just referred to.

2. The appropriation of $10.00 for the support of the Federation Placement Service, to be taken from the Institute treasury.

3. The appropriation when called for of $1.00 per member, from the Institute treasury, to meet the deficit in Publication Funds of the Federation.

4. The authorization of an annual appropriation to the Treasurer's office of $25.00 for clerical assistance.

President Carpenter announced the appointment of Dr. A. H. Smith as American Editor of Nutrition Abstracts and Reviews, in accordance with the request of the board of that journal. Doctor Smith reported on his activities to date. The Secretary reported the approval by the Council of $25.00 for clerical expenses and authorization of an additional $25.00 for the coming year.

The Secretary announced the recommendation of the Council that the annual assessment for 1941–1942 be fixed at $1.00. Upon motion of Dr. J. R. Murlin, it was voted to increase this assessment to $2.00, for the purpose of accumulating funds to purchase back the equity in The Journal of Nutrition, now held by The Wistar Institute.

Dr. E. M. Nelson reported informally for the Committee on Vitamin Nomenclature, stating that a name for Vitamin K was under consideration, and raising the question as to whether a new name for the present term "nicotinic acid" should be sought.

President Carpenter announced the appointment of the Nominating Committee for 1941–1942, as follows: G. O. Burr, Chairman, H. H. Beard, W. H. Chambers, H. J. Deuel, Helen Parsons.

The Tellers, R. M. Bethke and L. C. Norris, reported that the following were elected to the offices named:

President—A. G. Hogan
Vice-President—L. A. Maynard
Treasurer—W. H. Sebrell
Secretary—A. H. Smith
Councillor—H. B. Lewis
Editorial Board—C. A. Elvehjem, R. M. Wilder, G. O. Burr.

The Council recommended that the following be elected to membership:

Herbert E. Carter	Hazel M. Hauck	Robert R. Sealock
Charlotte Chatfield	T. B. Keith	Barnett Sure
Floyd S. Daft	Karl E. Mason	Ray D. Williams
Marianne Goettsch	Carl V. Moore	Robert R. Williams
J. R. Haag	George H. Satterfield	Dillworth W. Woolley
Robert S. Harris	Cecelia Schuck	

This recommendation was adopted, and the candidates mentioned were duly elected.

A vote of thanks was extended to the Local Committee of the Federation for the most excellent arrangements made for the meeting.

MEAD JOHNSON AWARD

The presentation of the Mead Johnson and Company Award for research on the Vitamin B complex for 1941 was made on behalf of the Institute by Dr. C. A. Elvehjem, a member of the Committee of Judges. The Award was presented to Dr. R. J. Williams, for his ''outstanding work in the discovery, isolation and identification of pantothenic acid.'' Doctor Williams made a brief response, describing some of his recent work.

EDITORIAL BOARD

During the period of 13 months since the last report (March, 1940 through March, 1941) 124 papers were published in The Journal of Nutrition; eighty-three submitted manuscripts were regarded as unacceptable, two being by foreign authors, and six papers were withdrawn by their authors. These, together with the eighteen now under consideration give a total of 231 papers considered by the editorial office and the board.

The objective of endeavoring to publish ten papers per issue, which means an average of ten pages per article, has been quite well achieved as shown by the following data:

> Vol. 19 (Jan.–June, 1940) .. . ... 57 articles
> Vol. 20 (July–Dec., 1940) ... . 60 articles
> Average per issue for year 1940 .. 9.9 articles
> Vols. 19 and 20 contained 613 and 635 pages, respectively.
> Average per article ... . 10.7 pages

Examination of the office records shows that the number of articles submitted has a seasonal character. For this reason it is quite difficult to secure as prompt publication as is desired at all seasons of the year.

The question whether the size of present volumes or the number of volumes per year can be increased in order to guarantee more prompt publication of accepted manuscripts quite obviously depends upon securing greater financial support for the Journal. The publisher, The Wistar Institute, has been quite generous in the matter of number of pages being printed per volume under the terms of our contract, the number per volume always having been more than the contractual allowance. It is to be hoped that the needed additional financial support can be obtained. One way that this might perhaps be done would be by the adoption of a more liberal attitude in the matter of acceptance of advertising of foods for human use. The editor and editorial board would welcome an expression of opinion in this matter by the Council and individual members of the society.

During the luncheon hour on April 16, 1941, a meeting of the editorial board was held at the Stevens Hotel. Editorial problems and policies were discussed.

Respectfully submitted,

L. A. MAYNARD, *Secretary,*

American Institute of Nutrition

ABSTRACTS OF PAPERS

Comparison of the anti-sterility and anti-muscular dystrophy potencies of alpha-tocopherol. Marianne Goettsch (introduced by W. M. Sperry), and A. M. Pappenheimer (by invitation), College of Physicians and Surgeons, New York.

Alpha-tocopherol has been reported to prevent resorption gestations in rats and mice; testicular degeneration in rats; nutritional muscular dystrophy in rats, guinea pigs, rabbits, dogs, ducks and mice; cerebellar degeneration in chicks; and spinal cord lesions in rats. The minimum amounts of alpha-tocopherol required by animals on vitamin E low diets are being investigated.

Anti-sterility potency. Single doses (0.5–50.0 mg.) of alpha-tocopherol were fed to rats at the beginning of the first gestation. Observations were made upon number of first, second and third litters born, number of young per litter, number born alive, number surviving 10 days of lactation and number protected against muscular dystrophy at the end of lactation. The requirement of mice was similarly determined.

Anti-muscular dystrophy potency. Suckling rats of females on E low diets were given single doses (0.3–3.0 mg.) of alpha-tocopherol during the period of lactation and the minimum dose for prevention of the disease determined.

Guinea pigs were cured of moderately severe nutritional muscular dystrophy, as determined by microscopic examination of muscle at biopsy, by single doses (25 mg.) of alpha-tocopherol. The lesions disappeared within 7 days and did not reappear for 3 to 4 months.

The alpha-tocopherol was administered orally or parenterally, in the form of natural alpha-tocopherol, syn-dl-alpha-tocopherol, or syn-dl-alpha-tocopherol acetate.

Dark adaptation of children in relation to dietary levels of vitamin A. Lydia J. Roberts, Helen Oldham (by invitation), Kathryn MacLennan (by invitation) and F. W. Schlutz, The University of Chicago.

Three groups of children from different socio-economic levels were tested and dietary studies were made on two of the groups. Fifty matched pairs of children were selected from the group whose subjects had the lowest vitamin A intake. One of each pair was given a vitamin A supplement while the other served as a control. Both control and experimental subjects were retested after 2 and 9 weeks of supplementation.

Analysis of the results show that:
1. The mean rod thresholds of the three groups of children known to have had widely different vitamin A intakes were almost identical.
2. Very few children in any group had subnormal adaptation as measured by this test.
3. There was no correlation between adaptometer readings and daily vitamin A intakes although a number of children were receiving less than 1000 I.U. per day in their diets.

4. A slight but significant improvement was found in the mean rod plateaus of the fifty children receiving supplementation when compared with those of their partners. Actual improvement was found in only eleven experimental as against six control subjects. The effect of the supplementation was most pronounced in the fourteen individuals whose original readings were subnormal.

These findings indicate either that the adaptometer does not measure the first signs of a vitamin A deficiency or that such a deficiency is uncommon among children in this area.

Significance of lead in foods. L. G. Lederer (by invitation) and F. C. Bing, Northwestern University School of Medicine, Chicago.

Lead is a widely distributed toxic metal that is found in minute amounts in almost every food if sufficiently sensitive methods for its detection are used. When the lead content of foods is higher than 1 p.p.m., it is our opinion that there is need for further study to determine if contamination is involved. The amount of lead in ordinary foods is such that it would be difficult for an infant to receive less than from 0.25 to 0.33 mg. of this element daily. These amounts are great enough to warrant taking all possible action to avoid contamination of foods with lead, especially of products which are fed to children. Milk, because of its high calcium content, tends to prevent the absorption of lead. Fat in the diet has no demonstrable effect.

Zinc retention in childhood. A. Stern (by invitation), Margaret Nalder (by invitation), and Icie G. Macy, Children's Fund of Michigan.

The polarographic method permits the easy and accurate determination of zinc in ash solutions of food, urine and feces. It was employed in the study of the physiological role of zinc in growth.

The average zinc content was found to be: for Irish potato, 0.077 mg./gm. dry weight; for mixed whole cow's milk, 2.89 mg./liter and in the daily composite, containing twenty-two individual foods, 15.6 mg. The average daily excretion in urine and feces of children amounted to 0.5 mg. (range 0.4 to 0.6 mg.) and 10.4 mg. (range 7 to 12 mg.) respectively. From 2.6 to 4.2 and 44.6 to 78.8% of the ingested zinc was excreted in the urine and feces, respectively.

The average daily zinc retention for eight children, 8 to 12 years of age, was observed. During 55 consecutive days for each child (eleven 5-day balances) totaling 440 experimental days the average daily zinc intake varied from 15.2 to 16.3 mg. (0.4 to 0.6 mg./kg. body weight) with an average retention of 4.8 mg. (0.11 mg./kg. body weight). The amount of zinc retained ranged from 17.0 to 52.3% of the zinc ingested, while the apparent absorption was 2.12 to 55.4%. The relation of zinc to iron, copper, manganese and nickel metabolism will be discussed.

The comparative effect of iron, protein, ascorbic acid and the vitamin B complex on hemoglobin formation in humans. Ruth M. Leverton (introduced by Helen S. Mitchell), and Alice G. Marsh, University of Nebraska, Lincoln.

The influence of daily supplements of iron, iron and ascorbic acid, certain vitamin B factors and protein on hemoglobin formation was determined in eighty college women. The subjects were matched according to their initial hemoglobin

value and then distributed equally into four groups. Group 1 received 120 mg. of iron from ferrous sulfate; group 2, 120 mg. iron plus 150 mg. ascorbic acid; group 3, 2 mg. thiamine plus 2 mg. riboflavin plus 25 mg. nicotinic acid; and group 4, 28 gm. protein from dry milk, cheese and peanuts.

At the end of 6 weeks the average rise in hemoglobin was 1.0, 1.2, 1.0 and 0.7 gm. per milliliter of blood for groups 1, 2, 3 and 4 respectively. For a second 6 weeks groups 1 and 2 were given only ascorbic acid, group 3 was given only thiamine and group 4, egg yolk. Values in all groups dropped but not as low as they had been at the beginning of the study.

These data, together with supplementary studies of iron absorption and excretion, emphasize the importance and value of dietary factors other than iron in overcoming the simple hypochromic anemia prevalent among women.

The influence of prenatal diet on the mother and child. J. H. Ebbs (by invitation), W. A. Scott (by invitation), and F. F. Tisdall, University of Toronto and Hospital for Sick Children, Toronto.

Studies have been made on the influence of good and poor prenatal diets on the health and obstetrical course of 400 women and their offspring. Each diet record was analyzed in order to determine as closely as possible the amount of the individual food components received by the mothers. Studies of the blood phosphatase, vitamin C and haemoglobin were made at intervals on both the mother and the child. Clinical observations and the laboratory investigations indicate that the prenatal diet has a striking influence on the health of the mother during pregnancy, labor and convalescence. The diet during pregnancy also markedly affects the health of the child during the first few months of life. The relation of the diet to the development of dental caries was also studied.

The influence of diet on the composition of weight gains in pre-school children. Jean E. Hawks (introduced by Marie Dye), and Gladys Everson (by invitation), Michigan State College, East Lansing.

On equi-caloric diets, pre-school children gained weight faster when they had 4 rather than 3 gm. of protein per kilogram. Milk, meat and whole egg as protein additions to the diet produced muscle, fat and bone growth because retentions of nitrogen and calcium as well as phosphorus, sodium, potassium and chlorine were increased. Egg white and gelatin as protein additions produced only muscle and fat growth because there was an increase in nitrogen storage, but no increase in calcium. At the same time magnesium and potassium retentions were lower, causing decreased minerals in the tissue. Thus milk, meat and whole egg may have produced a better type of weight gain than egg white and gelatin.

Additional calories from fat, starch or sugar produced tremendous weight gains which represented protein and fat tissue. The fat supplement caused no added calcium retention, therefore no increased bone growth. Carbohydrate supplements produced more nitrogen retention than fat and some calcium retention indicating protein, bone and fat growth. All high calorie diets reduced sodium, potassium and chlorine retentions. Thus calories added to a diet containing 3 gm. of protein per kilogram may be an economical way of producing muscle and fat growth, but, since the mineral content of the tissue is low, the weight gain may be less satisfactory than that produced by milk, meat and whole egg.

Prewar diet in Belgium: the influence of restriction of imports on the health of the population. L. Brouha (introduced by D. B. Dill), Harvard University, Cambridge.

The National Council for Nutrition in Belgium made a survey of the food problem during the period preceding the invasion. This nation-wide inquiry showed: (1) Average calories intake was around 2725 per day. (2) Proteins were sufficient, but animal proteins at the lowest limit. (3) Fats were at the lowest limit and butter was extremely low. (4) Carbohydrates were in excess. The diet was ill-balanced, the proportions being protein 1, fat 0.95, carbohydrate 5. Calcium and vitamin B were at the limit of danger. Vitamins A and D were low. The situation was already serious for the children. As a result of the war, suppression of importations, reduction of cattle and poultry, the actual diet is as follows:

1. Average calories: 900 to 1400. The deficit varies from 25 to 75%.
2. Proteins: 30 gm. Deficit from 25 to 85%.
3. Fats: 12 gm. Deficit from 30 to 90%.
4. Calcium: deficit increasing to 90%, between 3 to 18 years of age.
5. Vitamins are very low, for butter, milk, eggs, cheese, citrous fruits have practically disappeared. Milk is available only for children under 4 years.

It is beyond doubt that such an insufficient and unbalanced diet will have an ill effect upon the health of the population, mainly upon the health of children and adolescents.

What are the foodstuffs which would be most helpful in case some could be sent?

What is the diet that the population should have after the war is over in order to counteract the ill effects of partial starvation?

The alimentary interconversion of thiamine and cocarboxylase. E. S. Nasset and J. F. Waldo (by invitation), University of Rochester.

About half of a 74 micromol dose of thiamine or cocarboxylase is recoverable from a duodenostomy in the dog. When thiamine is fed, about 11% of the recovered portion is phosphorylated. Conversely, when cocarboxylase is fed, 55 to 78% is recovered as thiamine. The absorption and interconversion of these forms of the vitamin in the operated dogs occur rapidly in the upper portion of the digestive tract, including the stomach and the duodenum. Experiments done in vitro with various digestive juices in an attempt to localize the enzymatic processes concerned have been only partly successful. It has not been possible to demonstrate phosphorylation of thiamine in vitro with juices from the stomach, pancreas, duodenum or jejunum. Mixture of juices and a mucosa extract also were inactive. The hydrolysis of cocarboxylase to thiamine is readily effected by duodenal or jejunal juice.

The method of Melnick and Field was used in the determination of thiamine. Cocarboxylase was converted to thiamine with the aid of Taka-Diastase.

Studies of the excretion of thiamine and its degradation products in humans. H. Pollack, M. Ellenberg (by invitation), and H. Dolger (by invitation), Mt. Sinai Hospital, New York.

There are two components in human urine which stimulate the rate of glucose fermentation by yeast. One has definitely been isolated and identified as thiamine.

The other, not oxidizable in alkaline ferricyanide and not inactivated by bisulphite saturation, has been identified by inference as a pyrimidine. Schultz, Atkins and Fry have shown that the pyrimidines possess the same fermentation-stimulating activity as thiamine, mole for mole. Since the pyrimidine nucleus is an integral part of the thiamine molecule, it is important to determine the relationship of urinary pyrimidine to thiamine metabolism.

Evidence is presented to show: (a) Complete deprivation of dietary thiamine for a period of 10 days changed the urinary thiamine to pyrimidine ratio from approximately 9:1 to 1:9. (b) During this 10-day deprivation period the absolute amount of pyrimidine excretion remained at approximately the same level; while the free thiamine disappeared almost completely. (c) The 1 mg. "load test" before and after a 10-day deprivation period was not altered significantly. (d) The parenteral administration of 100 mg. of thiamine daily produced a tremendous increase in urinary pyrimidine excretion. (e) The deprivation of thiamine for 10 days did not give rise to any evidence of vitamin B_1 deficiency, either subjectively, or objectively as measured by electrocardiogram, blood sugar, cholesterol, total protein, hemoglobin, red blood count, blood pressure and pulse rate.

We wish to express our thanks to Dr. C. N. Frey and his associates. This work was supported in part by the Williams-Waterman Fund.

Further observations on induced thiamine deficiency in man; thiamine requirement of man. R. D. Williams (introduced by R. Wilder), and H. L. Mason (by invitation), The Mayo Foundation, Rochester, Minnesota.

Twelve healthy adult women were maintained for a period of 5 months on a rigidly controlled basal diet containing 400 to 450 μg. of thiamine. Factors of the vitamin B complex other than thiamine were supplied in the form of autoclaved brewers' yeast. This report will describe the mental state, the cardiovascular, gastrointestinal, metabolic and hematologic disorders observed during this period of moderate restriction of intake of thiamine and will compare them with observations made during a previous study,[1] already reported, in which subjects received a diet containing less than 150 μg. of thiamine.

Six of these subjects, maintained continuously on the controlled basal diet, were selected for the study of thiamine requirement of man. They were divided into two groups: Group 1 received thiamine chloride in an amount and for a period of time sufficient to replenish depleted stores of this vitamin; group 2 received only the basal diet. Thereafter, both groups of subjects received identical supplements of thiamine chloride in gradually increasing amounts. The mental and physical states of the subjects were correlated with the various levels of intake of thiamine and with the excretion of this vitamin in the urine.

[1] Williams, R. D., H. L. Mason, R. M. Wilder and B. F. Smith. Observations on induced thiamine (vitamin B_1) deficiency in man. Archives of Internal Med., vol. 66, p. 785, October, 1940.

Fat metabolism in rat acrodynia. F. W. Quackenbush (by invitation), F. Kummerow (by invitation), and H. Steenbock, University of Wisconsin, Madison.

Ethyl linolate was found to cure completely an acrodynia which had been produced in rats on a diet free from unsaturated fatty acids. Pantothenic acid

had no effect, pyridoxine alleviated the symptoms somewhat and a supplement of 'the two was more effective than either alone. Rice bran extract (Vitab) was still more effective but when fed for long periods did not prevent a remission with the appearance of scaly feet and tails. Further supplementation with either linoleic or arachidonic acid effected a cure; linolenic acid was not curative. Linoleic acid and arachidonic acid also promoted normal reproduction and satisfactory raising of the young.

Analyses of the total body fats revealed: (a) a reduction in the fat content and an increase in the iodine value with the production of dermatitis; (b) an increase in the fat content and a decrease in the iodine value after feeding linoleic acid; (c) a slight increase in the fat content but no decrease in iodine value after feeding pyridoxine; (d) a marked increase in fat content and a decrease in iodine value after feeding rice bran extract or pyridoxine and pantothenic acid.

The fatty acids from acrodynic rats or from rats which had been cured with rice bran extract were non-curative.

Fatty livers were not encountered.

Nicotinic acid and coenzyme levels in animal tissues. W. J. Dann and P. Handler (by invitation), Duke University Medical School, Durham, N. C.

Nicotinic acid in tissues has been measured by a new procedure (Proc. Am. Soc. Biol. Chem., vol. 35, p. li, 1941) and parallel V-factor (coenzymes I and II) estimations by the method of Kohn have been made. Results in two species are reported.

Mean figures for twelve albino rats follow: Coenzyme content, expressed as micrograms coenzyme I equivalent to 1 gm. fresh tissue, were liver 396 ± 33; kidney 650 ± 20; muscle 466 ± 9. Nicotinic acid content, micrograms per gram, liver 175 ± 3.7; kidney 132 ± 3.6; muscle 86 ± 0.9. All the nicotinic acid of muscle and probably all in kidney was present in coenzyme, but more than half the nicotinic acid in liver was not so combined. Direct nicotinic acid determinations on tissues of rats on a diet low in this factor gave rigid proof that the rat can synthesize this compound.

For ten normal dogs tissue contents of coenzyme were: liver 438 ± 46; kidney 504 ± 18; muscle 310 ± 12. Nicotinic acid contents were: liver 153 ± 12; kidney 95 ± 3.4; muscle 71 ± 1.9. All the nicotinic acid of kidney is combined in coenzyme but this is not the case in liver and muscle. In blacktongue the liver content decreases to 42% of normal and that of muscle to 72%; kidney content is unchanged. The liver nicotinic acid not bound in coenzyme is scarcely decreased at all.

The sources of the wide discrepancies between the results of different workers will be discussed briefly.

Factors affecting the increase in blood plasma ascorbic acid after oral ingestion of vitamin C. E. Neige Todhunter, State College of Washington, Pullman.

In mass studies of vitamin-C nutrition it is not always possible to obtain fasting blood samples. This study was undertaken primarily to ascertain how rapidly and to what extent ascorbic acid with breakfast would cause a rise in plasma ascorbic acid above the fasting level; also to study the effect of different

food sources of the vitamin in raising the plasma level, and to gain some further knowledge of the metabolism of vitamin C. Ascorbic acid was determined by Farmer and Abt micromethod and by a photoelectric colorimeter method. Blood samples were taken at 7:50 A.M., 8:30 and each succeeding $\frac{1}{2}$ hour till noon. At 8:00 A.M. 50 mg. ascorbic acid, either crystalline, or in a test food was taken with a standard breakfast. Data for each subject (college women) were collected for 3 days for each test food, and good agreement was obtained. All subjects showed fair agreement in time of attaining the peak of the absorption curve. The amount of increase in plasma level was influenced by the initial level, and the hour of maximum increase depended on the source of the vitamin, that from strawberries and cauliflower being later than from orange juice, whole orange or crystalline ascorbic acid. Within 3 to 4 hours after the meal, blood values returned to the fasting level. Iron salts, in either ferrous or ferric form, did not appear to delay absorption, nor did a high fat intake. Increased intakes of 100, 200 and 400 mg. of crystalline ascorbic acid gave corresponding increases in the peak of the plasma absorption curve.

The influence of certain organic compounds on perosis. T. H. Jukes, University of California, Davis.

Perosis was produced in chicks by a diet of glucose, washed casein, gelatin (or creatine), yeast, gum arabic, salt mixture, soy bean oil, and fish oil. Perosis was prevented and the growth rate was doubled by the addition of choline. However, if manganese was omitted from the salt mixture, choline did not prevent perosis. Manganese and choline are thus both necessary for the prevention of perosis in chicks as well as in turkeys.

A number of choline derivatives, supplied by Dr. A. D. Welch, were studied for their effects on perosis and growth. The triethyl homologue of choline was neither anti-perotic nor growth-promoting, while the monomethyl-diethyl homologue prevented perosis but did not promote growth. Betaine was completely ineffective, but betaine aldehyde had weak anti-perotic and growth-promoting effects similar to those observed on insufficient levels of choline. This may indicate a partial conversion of betaine aldehyde to choline in vivo. Furthermore, the failure of monomethyl-diethyl choline to promote growth while preventing perosis (a result also obtained with turkeys) may indicate that the growth-promoting and anti-perotic properties of choline reside in different parts of the molecule.

If gelatin (or creatine) was omitted, perosis was not produced by the basal diet. This contrasts chicks with turkeys, which develop perosis even when gelatin (or creatine) is omitted from a similar diet.

Isolation of lanthionine from various proteins. D. B. Jones and M. J. Horn (by invitation), Bureau of Agricultural Chemistry and Engineering, United States Department of Agriculture, Washington, D. C.

Recently there was described by the authors the isolation from acid hydrolysates of sodium carbonate-treated wool of a new thio ether diamino acid, lanthionine, $HOOC.CH(NH_2).CH_2.S.CH_2.CH(NH_2).COOH$. By following the same procedure as was used with wool, we have now found that this amino acid can be isolated not only from other keratins, such as hair and feathers, but also from lactalbumin.

Lanthionine has also been isolated from the hydrolysis products of wool that had been treated with dilute sodium sulfide solution in the same manner as with sodium carbonate. Apparently the reagents used cause a splitting of the disulfide bonds of cystine resulting in structural changes of the protein so that on subsequent hydrolysis a thio ether amino acid is formed. It appears probable that the formation of lanthionine under these conditions may well be a property common to all proteins containing cystine. It is possible that the cystine of dietary proteins may be converted into lanthionine in the intestinal tract, which at times is distinctly alkaline in reaction. That a thio ether amino acid may play an important part in the sulfur metabolism of animals has already been assumed in connection with the possible intermediary relationship of cystine and methionine. Feeding experiments are in progress in order to see whether lanthionine can replace cystine or methionine in the diet.

Nutrition in carcinogenesis. C. P. Rhoads (introduced by W. T. Salter), and C. J. Kensler (by invitation), The Memorial Hospital, New York.

Experimental evidence has been obtained that hepatic cancer in three different strains of rats can be produced regularly by the administration of dimethyl amino azobenzol to rats taking a diet of brown rice and carrots. Supplement of this diet with yeast or liver extract in adequate amounts prevents completely the development of the cancer. The protective factor is none of the constituents of the vitamin B complex so far described. Certain evidence as to its nature will be presented.

The effect of feeding the carcinogen to animals taking the deficient diet is an inhibition of the activity of at least two enzyme systems, co-carboxylase and cozymase, thus producing a conditioned or secondary deficiency disease. The metabolic breakdown products of the carcinogen have been isolated from the excreta of the treated animals and their structure established. One of these products is profoundly inhibitory to both cocarboxylase and cozymase systems in vitro. Acetylation of this compound, which occurs in vitro, removes all toxicity.

The development of the mutation which characterizes the malignant tissue is marked by the presence of an oxidizing enzyme system which is no longer susceptible to the inhibitory effect of the toxic metabolic product of the carcinogen. The oxidation of the normal liver from which the cancer is derived is extremely susceptible to inhibition by this compound. This is the first demonstration that cancer tissue induced by a known chemical possesses an oxidative system which is immune to that chemical or its metabolic product.

Progressive iodination of serum albumin. W. T. Salter and Jytte Muus (by invitation), Thorndike Memorial Laboratory, Boston City Hospital.

When horse serum albumin is iodinated in alkaline solution, it assumes such marked thyroidal activity that it becomes a useful hormone with which to treat human myxedema. When the iodine is added to the protein in stages, at first no thyroidal activity is encountered—up to about 6% iodine content. This stage corresponds to iodination of the tyrosine in the protein. Thereafter thyroidal activity steadily increases as more iodine is added up to about 11%. This iodine moiety is greater than can be accounted for by the histidine present. In the course

of this latter treatment a thyroxine-like fraction is developed, which separates on hydrolysis. Thus simple alkaline iodination of a circulating protein molecule transforms one or more of its constituent amino-acids into thyroid hormone.

This finding is of special interest because the circulating ''hormonal'' iodine of blood plasma is found to be prominent in the albumin fraction. This circulating albuminous iodine also consists of a thyroxine-like and a non-thyroxine fraction.

ABSTRACTS OF PAPERS READ BY TITLE

Vitamin A metabolism of college students. Jane C. Ebbs (by invitation and Esther L. Batchelder, Rhode Island Agricultural Experiment Station, Kingston.

Dark adaptation records of 223 healthy college students (approximately one-fifth of the college enrollment) have been measured and analyzed statistically. The instrument used for the tests was the ''rhodometer'' previously reported. A bell-shaped distribution curve has been attained (skewness of 0.3315) for the minimum brightness perceived at the end of a 15-minute test. The mean, median, and mode were log 5.28 ± .045, log 5.34 and log 5.40 micro-microlamberts, respectively.

Several studies have been made of the response to massive doses of vitamin A. The first observations were of the dark adaptation 24 hours after administration of the test dose. One subject who had shown a gradual decrease in dark adapting ability which eventually fell below that of any of the subjects tested in the campus survey was tested each hour for 6 hours after administration of 91,000 I.U. as halibut liver oil. After 1 hour no apparent change in dark adaptation was observed; after 2, 3, 4 and 5 hours progressive increase was observed, reaching log 5.35 micro-microlamberts (the median value of the campus survey) after 5 hours. This value was maintained in tests given 24 hours following taking of the massive dose. It was maintained for several days on an intake of 91,000 I.U., but no further increase was observed.

Prophylactic requirement for alpha-tocopherol in male and female rats. Gladys A. Emerson (by invitation), and H. M. Evans (by invitation), University of California, Berkeley.

Alpha-tocopherol acetate was administered at three levels six times weekly to rats held on an E-low ration; the levels were 0.1, 0.25 and 0.75 mg.

E-low animals of both sexes showed the typical plateauing in weight characteristic of vitamin E deficiency. Growth of all tocopherol-fed rats exceeded that of natural-food-fed rats.

Twenty-five hundredths milligram α-tocopherol was sufficient for normal reproduction in the male, while 0.1 mg. was inadequate as evidenced by the fact that half the copulations with normal females were sterile.

The number and weight of young born to the females in each tocopherol group were the same. A striking difference in the young appeared in the lactation period. The offspring of 0.1 mg. mothers exhibited the dystrophy typical of vitamin E deficiency; half were dead by the thirtieth day and most of the survivors exhibited some degree of paralysis.

The young of the mothers receiving 0.25 mg. showed the same incidence but a less severe dystrophy, all affected animals having recovered by the thirtieth day.

No dystrophy occurred in the young of mothers receiving 0.75 mg. and it is notable that their average weight at 30 days (92 gm.) was 9 gm. higher than in the offspring of mothers on the natural food diet.

A second litter series gave almost identical results.

The influence of sex on iron assimilation in the rat. S. W. Kletzien, New York State Institute for the Study of Malignant Disease, Buffalo.

Various published reports on the influence of sex on iron assimilation convey the impression that the female organism more efficiently assimilates iron than the male. Our data based on iron analyses of the total blood, liver, spleen, and carcass do not bear out this impression. We have found, as have others, that the female responds more quickly with a higher concentration of hemoglobin and exhibits evidence of greater liver iron stores than the male, but presumably these result from the lesser requirements for growth in contrast to those of the male. The male animal puts out a larger volume of blood of a lower hemoglobin content and builds into its soft tissues more iron on the other hand, thereby off-setting any apparent gains in the female and actually exceeding them in total iron assimilated.

Carrying our studies further we have found that ovariectomy increases iron assimilation as does castration; such procedures nullify any previous differences in the pattern of iron distribution and bring about comparable responses in both sexes. Pregnant females as compared with virgin litter mate controls and males reveal however an increased capacity to assimilate iron on similar iron, copper, and basal diet intakes. This in itself is however not sufficient to maintain pre-pregnancy stores of iron. The iron assimilation of the pregnant female thus simulates that of the rapidly growing male.

Calcium balances of young women on their customary or self-chosen diets. Hughina McKay (by invitation), M. B. Patton (by invitation), Margaret A. Ohlson (by invitation), Martha S. Pittman, Ruth M. Leverton (by invitation), and G. Stearns, Agricultural Experiment Stations of Iowa, Kansas, Nebraska and Ohio.

Calcium balance studies of 109 college women, following their customary mode of living, eating their customary diets, and presumably in good health, showed a mean daily intake and balance of 0.9538 and 0.0307 gm. respectively. Corresponding figures for another young woman studied for forty-five 5-day periods were 1.2389 and 0.0711.

Thirty-six of the large group were studied for two or more periods. The variability of the ''repeat'' group was similar to that of the one individual.

Average daily intakes for the 110 women ranged from 0.322 to 2.323 gm. One-third of the women having intakes less than 0.699 were in positive balance, as compared to two-thirds of the women having intakes greater than 0.699.

There was a trend toward more efficient utilization of calcium by women from 17 to 20 years of age than by older women.

Treatment of the data by linear regression indicates that, in 95% of the predictions for a population of which the group of 109 is representative, positive calcium balances will occur on intakes ranging from 0.758 to 0.895 gm. daily.

The relation of pantothenic acid and succinic acid to the growth of certain micro-organisms. L. Rane (by invitation), and Y. Subbarow (by invitation), Massachusetts Department of Health and the Harvard Medical School, Boston.

Micro-organisms have been used as biological reagents to isolate pantothenic acid. Strains of group A hemolytic streptococcus and types of pneumococcus require the whole conjugated compound for growth. The β-alanine fraction is an essential growth factor for certain strains of the diphtheria bacillus while the acid portion is necessary for some strains of group D hemolytic streptococcus.

Fractionation of a highly purified liver extract yielded a crystalline compound, the activity of which in promoting the growth of a strain of group D hemolytic streptococcus was comparable to that of hydrolyzed and non-hydrolyzed pantothenic acid. The compound was identified as succinic acid. Synthetic succinic acid was similarly active when large inocula of organisms were used to seed the test media. Light inocula capable of inducing growth with the natural compound failed to show any activity with the synthetic. This variation indicated the possibility of a slight contamination of the natural compound by pantothenic acid or a component of pantothenic acid. The addition of pantothenic acid in completely inactive concentrations to synthetic succinic acid produced full growth of even small inocula.

Further discussion will be made of the possible nutritional significance of combinations of compounds in sub-optimum proportions.

Studies on the specificity of inositol. D. W. Woolley (by invitation), The Rockefeller Institute for Medical Research, New York.

Since it has been shown that inositol cures alopecia in mice fed on a purified diet, it was of interest to determine if other related substances possessed similar activity. In order to compare the responses of widely-separated species and to improve the quantitative aspects of the technique, a species of yeast was also tested for its behavior towards the compounds. For the yeast inositol was active; phytin, sodium phytate, inositol hexaacetate, soybean cephalin, quercitol, d-inositol, d-inositol monomethyl ether, l-inositol and l-inositol monomethyl ether were less than one-tenth as active as inositol. Mytilitol (methyl inositol) was active. The chief difference found between the responses of yeast and mice was the ability of mice to respond to esters of inositol. Thus phytin, inositol hexaacetate and soybean cephalin were active in mice.

Pantothenic acid has also been found to influence alopecia in mice. Hair may be lost in the absence of inositol or of pantothenic acid. In alopecia which results from lack of inositol, eventual spontaneous cure (either partial or complete) may occur.

Nutritional status of college women; factors contributing to variability in basal metabolism. Charlotte M. Young (by invitation), Martha S. Pittman, Elva G. Donelson (by invitation), and Gladys M. Kinsman (by invitation), Kansas State College, University of Minnesota, Oklahoma Agricultural and Mechanical College and Iowa State College. (Approved for publication by the Advisory Committee of the Regional Project of the North Central States Relation to the Nutritional Status of College Women.)

At least four basal metabolism observations are reported on more than 400 college women from one university and three colleges. About half of the students were observed for 2 to 4 years. Thus both a longitudinal and a cross sectional study have been possible.

The purpose of the study was to record the basal metabolism of the college woman measured under her usual living conditions. Variability was considered a fundamental characteristic of basal metabolism. Certain factors contributing to variability have been segregated by statistical methods.

Standard deviations for inter- and intra-individual variabilities confirmed the work of Boothby and Berkson (Am. J. Physiol., vol. 121, pp. 660–683). Data have been obtained to show that the method of selection of data is a variability factor of importance. The procedure of choice is (1) a careful control of the testing environment and (2) use of all data except that discarded at the time of the test for errors in technique or non-basal condition of the subject. Significantly more variable results were obtained on the first day of testing which suggests the use of one "practice day" for adaptation to the testing routine. Increased scholastic or extracurricular responsibilities were found to increase the variability of basal metabolism. Certain other factors are being investigated.

GROUP DISCUSSIONS

7: 30 P.M.

A. Making nutrition function. Helen Mitchell, presiding.

Dr. Helen Mitchell, chairman, stated that the standards for specific nutrients had been set up in 1925 by the League of Nations and are now being revised. Dr. Lydia Roberts, member of the Committee on Food and Nutrition of the National Research Council, presented the tentative revision, pointed out the problems encountered in trying to establish values, stated the bases for selection of proposed standards, and how to meet them with a diet of natural foodstuffs. Dr. E. V. McCollum considered that the thiamine, riboflavin and ascorbic acid levels are too generous, the iron and vitamin D levels too low. Dr. R. M. Wilder defended the thiamine figures and Dr. W. H. Sebrell those for riboflavin. Doctor Wilder then gave the history of the enriched flour program, concluding that since food fortification is here to stay we should fortify the foods used in large amounts by the poorer classes. Dr. Muriel Brown explained the use of nutrition clinics in Tennessee as part of the defense program. They hope to teach the people to desire to improve

their food habits by means of a cooperative health program for the undernourished children in the community. Dr. Martha Koehne emphasized the need to inspire people with the desire to be better than they are, to give them the information to make them better and to help them to apply this information. Colonel Paul Howe discussed the feeding of the army, and methods used to secure for the men menus that are nutritionally adequate and to have the food properly prepared.

B. Trace elements in nutrition. C. A. Elvehjem, presiding.

The significance of trace elements in nutrition was summarized by seven different speakers and after each speaker there was considerable informal discussion. M. O. Schultze reviewed the recent work on iron and copper with emphasis on the forms of iron in the body and the action of copper in the production of these compounds. E. Hove discussed both zinc and boron and presented ample evidence for the essential nature of zinc, but stated that clear-cut evidence for the necessity of boron in animal nutrition was still not extensive. We were fortunate in having present in our group W. G. E. Eggleton of Lester Institute, Shanghai, China, and he discussed briefly his recent work on zinc. A very complete summary of all our knowledge regarding cobalt and manganese was given by J. M. Orten. The role of cobalt in both the prevention of anemia and the production of polycythemia stimulated considerable discussion. The so-called toxic trace elements, fluorine and selenium, were discussed by P. H. Phillips and A. L. Moxon. Phillips emphasized the importance of maintaining the fluorine content of all diets at a low level in spite of certain experimental results indicating that fluorine retards dental caries. Moxon described recent studies on methods of counteracting selenium poisoning. C. V. Moore gave an excellent summary of the clinical aspects of the trace elements, and showed the limitations of our knowledge regarding the importance of these elements in human nutrition. The final discussion centered around the variable results obtained with different species, especially in regard to studies on the availability of iron.

C. Nutritive significance of individual carbohydrates. H. J. Deuel, Jr., presiding.

Phosphorylation and the comparative absorption of various sugars.

G. T. Cori. When the rate of phosphorylation is compared with the rate of absorption it is found that the two mechanisms do not proceed at a parallel rate. For instance galactose is the most rapidly absorbed of the sugars studied, followed in turn by glucose and fructose. On the other hand fructose is the best phosphate acceptor with galactose the poorest. Iodoacetic acid and phlorhidzin poisoning affect the organism in somewhat different ways. Phosphorylation is not abolished by adrenalectomy as the phosphorylation of some of the vitamins can occur under this condition.

The relative glycogenic and ketolytic action of the various sugars.

J. R. Murlin. The conclusion seems evident, even though the ketonuria is produced by such widely different experimental procedures as phlorhidzin poisoning, a high fat diet, fasting in humans, or feeding four carbon atom fatty acids to rats, that the individual carbohydrates vary in their ability to reduce an acetonuria. In general the results may be summarized in that galactose or fructose are markedly superior to glucose, with lactose and sucrose occupying intermediate positions. Galactose seems to form a glycogen more resistant to breakdown than that formed after glucose.

Respiratory metabolism.

T. M. Carpenter. When heat production was used rather than surface area as a basis of sugar feeding, a better correlation was observed betwen different species. After fructose feeding a similarity is noted in the R.Q. of the canary, the goat, monkey, and man. The rat behaves differently. After galactose feeding man responds in a manner different from all other species studied. After ingestion of glucose the monkey and man behave similarly. The R.Q. of the canary increases very markedly. In the cat the R.Q. continues to rise for a long period.

Metabolism of hexitols.

Fred W. Ellis. In a comparison after feeding mannitol and sorbitol it was noted that sorbitol was well tolerated while mannitol was not. When fed in cocoa butter over a 2–3 day period sorbitol did cause a slight increase in liver glycogen while mannitol failed to elicit such a response.

Citric acid metabolism.

A. H. Smith. Citric acid is always produced in metabolic processes. In man from 0.20–1.00 gm. per day is eliminated. The origin of citric acid is as yet unknown, although the effects of various foods and regimes have ben studied. Thus when bicarbonate is fed the citric acid output is increased. The ingestion of acid causes the opposite effect. Of the various foods carbohydrate causes the greatest output of citric acid, although there is a variation in the response to the individual sugars. Sucrose, starch, and dextrin are about twice as effective as glucose, fructose, and galactose.

D. Effect of heat upon the nutritive value of proteins.
Agnes Fay Morgan, presiding.

Various phases of the effect of heat upon proteins were discussed by Drs. D. Breese Jones, L. C. Norris, J. W. Hayward, Helen Parsons, Joseph Routh, H. A. Mattill, R. J. Block, and A. G. Hogan. Several others also participated in the discussion.

Among the many points brought out was the fact that moist heat greatly improves the digestibility and the biological value of many plant proteins. Dry

heat may also improve the biological value of soybean meal but temperature and exposure time are more important than the type of cooking. This is also true for fish meals. Other animal proteins, meat products, when heated, have a decreased biological value not due to destruction of lysine and histidine since the heated protein when hydrolyzed is comparable with the unheated hydrolyzed protein. Because the loss in the biological value of wheat, casein, edestin, and liver, caused by dry heat, can be restored by either hydrolysis or additional lysine, it was suggested that dehydration results in protection of the lysine against enzymatic reactions. The addition of valine and leucine to lactalbumin made appreciable improvement in the biological value lowered by heating. Dogs on diets containing heated casein at death showed fatty livers and reduced serum protein. Neither additional lysine and histidine nor an increased intake of the protein compensated for the heat injury. It was suggested that new methods of studying the molecular structure of protein must be sought to unravel the story of its biological value. The significance in human nutrition of the deleterious effects of heat upon most animal proteins was emphasized.

E. Heat regulation. L. H. Newburgh, presiding.

Dr. R. L. Day emphasized that the premature infant had a higher heat loss than the adult because of a much greater proportionate surface area and for other reasons. Diurnal variations in body temperature were absent in early infancy. He pointed out that body temperatures as low as 88°F. were compatible with apparently normal health in premature infants. Dr. H. G. Barbour was concerned with the mechanism of control of loss of heat. He showed that in a cold environment or when the internal temperature is rising at the beginning of a fever, heat is retained because vaporization of water is diminished. This diminution is brought about by concentration of the blood, the result of the passage of water out of the blood. It can be shown that there is a general increase in intra-cellular water content at this time. With an increase in environmental temperature there is a dilution of the blood before the increase in vaporization. The control of regulation of internal temperature appears to lie in the hypothalamus; cooling anteriorly, heating posteriorly. Dr. J. D. Hardy pointed out that there are two types of fever. One is due to a dramatic increase in heat production, and the other is due to a slight to moderate increase in heat production with an active suppression of heat loss. The first type occurs with exercise or a chill and may occur in health or disease, whereas the second type occurs in disease. Quantitative aspects were brought out by means of lantern slides. Dr. D. B. Dill spoke about acclimatization to high environmental temperatures. He pointed out that there was a large individual variation in capacity to adapt to high temperatures. A great variation occurs in individuals to sweat and also in the composition of sweat. When first exposed to such temperatures the sweat is concentrated, but later it becomes diluted. Dr. J. A. Greene described a method for measuring the per cent of heat lost by vaporization of water in the rat in the basal state and throughout the 24 hours. He also compared the per cents of heat dissipated by vaporization on the part of various mammals and noted that the albino rat lost 22%, the marmot 20%, the elephant 19%, the cow 26%, the rat 24% and the rabbit 25%.

F. Comparative nutritional requirements of various species. C. M. McCay, presiding.

A brief introduction showing the interplay between insect and animal nutrition was given by the chairman. Dr. R. Wulzen reviewed briefly her many years of study of the nutrition of planaria. Dr. W. H. Peterson then talked about the recent advances in determining the vitamin requirements of bacteria. Dr. Marianne Goettsch reviewed the history of the discovery of vitamin E and its importance to many animal species in the prevention of muscular dystrophy, and Dr. K. Mason summarized recent knowledge of sterility in the male. Dr. F. C. Bing presented an interesting paper concerning the usefulness and limitations of the mouse for experiments in nutrition. From the floor, Dr. C. A. Mills presented data showing the higher requirement of mice for thiamine when kept in a warm room. The program was concluded with a comparison by Dr. I. McQuarrie of the diseases of children and adults observed in China with those of similar people in America.

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